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A COMPARATIVE STUDY OF INTESTINAL STREPTOCOCCI FROM THE HORSE, THE COW, AND MAN.*

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INTRODUCTION.

THE first real clue to the systematic relationships of the streptococci was furnished by Gordon when he pointed out that these organisms could be separated into well defined groups by a study of their fermentative reactions in various carbohydrate media (Gordon, 1904; Gordon, 1905). Houston carried forward the application of Gordon's tests (Houston, 1905; Houston, 1906); and finally Andrewes and Horder (1906) founded upon all these results a rational classification of the genus into seven main types or species. The basis of their division was statistical or biometrical. It involved the examination of records of many individual cultures (1,200 in the case of the streptococci) and the study of the frequency with which various characters, or combinations of characters, occurred. Type centers, according to the statistical method, are defined by the occurrence of a large number of individuals with a given characteristic. These common types, among such variable organisms as the bacteria, may properly be considered as representing species, about which the rarer varieties are grouped.

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The chief characters of the species of streptococci thus defined by Andrewes and Horder are indicated concisely in the table below (Table 1). Strept. equinus is described as characteristic of the intestine of the herbivora. It was abundant in horse-dung and was the commonest form in the street air of London. All the other forms were primarily of human origin, Strept. mitis and Strept. salivarius from the normal throat, Strept. fecalis from the normal intestine, and Strept. pyogenes and Strept. anginosus from diseased conditions.

TABLE 1.

Tabular Classification of the Streptococci.
(Andrewes and Horder, 1906.)

Species	Characteristics								
	Milk Clot	Neutral Red	Saccharose	Lactose	Raffinose	Inulin	Salicin	Coniferin	Mannit
Strept. equinus	−	−	+	−	−	−	+	+	−
" mitis	−	−	+	+	−	−	+	−	−
" pyogenes	−	−	+	+	−	−	+	−	−
" salivarius	+	+	+	+	+	−	−	−	−
" anginosus	+	+	+	+	+	−	−	−	−
" fecalis	+	+	+	+	−	−	+	+	+
Pneumococcus	+	−	+	+	+	+	−	−	−

One of the most interesting practical points about this classification lies in the fact that all the six streptococci of human origin ferment lactose, while Strept. equinus, which Andrewes and Horder hold to be characteristic of the intestine of the herbivora, fails to do so. If a general distinction between human and animal streptococci could be demonstrated it would prove of much practical, as well as theoretical importance. Sanitarians are continually seeking, and seeking in vain, for some criterion by which sewage pollution of human origin may be distinguished from the surface wash of streets and agricultural land. The colon bacilli from the intestines of various warm blooded animals have been shown in many investigations—the latest and most exhaustive coming from Dr. Bettencourt's laboratory at Lisbon (Ferreira, Horta, and Paredes, 1908; Bettencourt and Borges, 1908*a* and 1908*b*)—to be identical. If the streptococci should prove to be characteristic it would greatly aid the water analyst.

The work of Gordon, and Andrewes and Horder, to which reference has been made, pointed to lactose as the best differential medium

for distinguishing human and animal streptococci. These investigators showed clearly that a large proportion of streptococci of human origin ferment lactose while most of those derived from horse-dung or street air fail to do so. On the other hand Houston (1906) in the examination of cow-dung obtained widely different results. Eighty-five per cent of his cultures from this source fermented lactose; but their reactions in mannit, raffinose, and neutral red were somewhat characteristic. Mannit was fermented by 24 per cent of the human cultures and by none of the bovine strains; raffinose was fermented by 32 per cent of the human and by 74 per cent of the bovine cultures; neutral red was reduced by 39 per cent of the human strains and by none of those from cow-dung.

These results suggest that both the horse and cow have characteristic types of intestinal streptococci which differ from each other and from those commonly found in the intestine of man. Our object in the present investigation was to confirm this conclusion by the study of a new series of cultures from the three sources. In particular we wished to obtain quantitative results with regard to the amount of acid formed in various media. This aspect of the question has been ignored by all the English observers, who are content merely to classify their cultures as fermenting or non-fermenting. Recent studies of the Coccaceae as a whole have shown however that exact quantitative data are of much value in classification (Winslow, 1908). We chose dextrose, lactose, raffinose, and mannit as the carbohydrates to be tested. Dextrose serves as a type of the simple sugars which are fermented to some extent by almost all streptococci. Lactose and raffinose represent, respectively, the aldehydic and anhydric groups of sugars. Mannit represents the alcohols. Three hundred and two strains of streptococci, from human, equine, and bovine feces, were grown in broths containing these carbohydrates and the resulting acidity in each case was determined by titration. The results as analyzed below indicate definite and characteristic differences between the streptococci from the three species of animals.

Methods.—All the streptococci studied were isolated from fresh feces, collected in a sterile tin can with tight-fitting cover. Human feces were deposited directly in the can while horse-dung and cow-dung were taken from the stable floor within a few minutes after ejection. Streptococci were isolated from 15 samples of human feces, 12 samples of horse-dung, and 22 samples of cow-dung. In all cases care was

taken to remove the outer layer of the feces and to take samples from the interior in order to avoid contaminations from stable floor or air.

The attempt was made at first to isolate the streptococci by inoculating a loop of feces into dextrose broth, incubating for various periods from 4 to 96 hours, and then plating on agar. This procedure is effectual in isolating streptococci from polluted water; but with feces it utterly failed in our hands. Spore-forming bacilli were found upon the plates or no growth appeared at all. It is probable that the streptococci were overgrown by other bacteria, perhaps by forms like B. acidophilus.

We therefore adopted the method of direct plating on agar without any preliminary enrichment whatever; and this proved generally successful. Two or three heavy platinum loopfuls of feces were well shaken up in 10 c.c. of sterile water and two or three loops of the resulting suspension carried over to a tube of melted agar. This was poured into a Petri dish and incubated at 37° for 24–48 hours. Agar streak cultures were made from minute round colonies on these plates and the appearance of these streaks after 24–48 hours at 37° was generally characteristic. Streaks showing a faint veil-like growth or a few dotted colonies or a thin line confined to the streak itself were almost always streptococci. In isolating the first 210 strains a microscopic examination was made. Smears were stained for 30 seconds with cold carbol fuchsin and cultures showing chains, pairs, or single cocci were accepted. In every one of these 210 cases the microscopic examination confirmed the conclusion based on the macroscopic appearance; and we are confident that the characteristic growth of the streptococci can be easily recognized by the experienced observer. Heavy growths on the streak always proved to be bacilli. Chromogenic, vigorously growing staphylococci were not found at all.

Streptococci were least easily detected on the plates made from cow-dung. The latter showed a great many small white colonies from which selection was difficult. Four out of five cultures streaked from cow-dung proved to be bacilli while not one out of five of the cultures isolated from horse-dung as streptococci failed to prove so.

A rough estimate was made of the total number of bacteria in one sample of each of the three kinds of feces, by plating high dilutions on agar, and incubating at 37° for 48 hours. The number of streptococci was estimated by isolating and identifying colonies which seemed characteristic. The sample of human feces showed an average of 8 million bacteria per gram of wet feces and 2,500,000 were streptococci. The proportion of streptococci is somewhat higher than that reported by MacNeal, Latzer, and Kerr (1909) who found that streptococci made up from 10 to 20 per cent of the total bacteria present. This is perhaps because MacNeal and his associates determined their total count by direct enumeration under the microscope, while our "total" is only the agar count at 37°. It is probable that most of the intestinal cocci grow on ordinary laboratory media, while we know that many bacilli and spirilla do not. In the sample of the horse feces we found an average of 3 million bacteria, of which nearly half were streptococci. This corresponds with our general experience of the high proportion of streptococci in equine feces. Cow-dung on the other hand showed in the sample examined an average of 100 million bacteria per gram of wet feces, of which 10 million were streptococci. This small ratio of streptococci (altho the actual numbers were high) explains the difficulty experienced throughout in isolating streptococci from cow-dung.

An interesting individual case was met with in the study of human feces, in which streptococci could rarely be found at all and in which all bacteria growing on ordinary

media were present in comparatively small numbers. Altogether the feces of 11 different persons were examined and streptococci were easily isolated in 10 cases. From this one individual, 7 samples were taken and 67 plates were made by the usual method (a loopful or two from feces to 10 c.c. water and a loopful or two of water to a plate). Only 22 out of the 67 plates showed growth and but 6 showed streptococci.

By the methods described above 302 cultures of streptococci were obtained in all, 116 from man, 100 from the horse, and 86 from the cow. As soon as a streak proved positive it was inoculated into broth tubes containing the four carbohydrate media tested. These were incubated for 72 hours at 37° and then titrated. Five c.c. of the liquid in each tube was measured out into a graduate, mixed with 45 c.c. of distilled water, and titrated in the cold against $n/20$ NaOH, using phenolphthalein as an indicator. Blank titrations were made at the same time on sterile tubes of the same batch and their results subtracted from those obtained for the inoculated tubes. The media used were in all cases made up with one per cent Witte's peptone, 0.25 per cent Liebig's beef extract, and one per cent of the carbohydrate to be tested.

Positive acid reactions were generally associated with obvious appearances of growth in the form of profuse white sediment along the bottom and sides of the tubes. In a very few cases, however, acid was produced in tubes which appeared clear to the eye.

In the case of negative tubes, with no rise in acidity and no turbidity or sediment the lack of development might conceivably be due to a failure of inoculation. Great care was taken however to transfer as much of the cultures as could be carried on a platinum loop. The fact that obvious growth almost always occurred in the dextrose broth tubes shows pretty clearly that inoculation was not at fault. The contents of 49 of the clear tubes of various media were examined by plating out on agar. Three tubes showed many colonies, 15 showed 1 to 6 colonies, and 31 showed none. It may reasonably be assumed that in such cases the streptococci introduced had simply failed to develop and gradually died out on account of the lack of suitable carbohydrate pabulum, upon which these organisms appear to be highly dependent.

RESULTS.

The results of the individual titrations are given in tabular form at the end of the paper. The first column indicates the culture examined, and the second, the number of the sample of feces from which it was derived. The figures for acidity represent the difference between the value obtained for each culture and the value of an inoculated control incubated under similar conditions. The results are probably significant within 0.2 per cent acidity.

A clearer idea of the meaning of these results may be gained from an inspection of Table 2 in which they are grouped together into acidity-classes, and from Charts 1, 2, 3, and 4, which have been plotted from the values in Table 2.

Dextrose is fermented more or less vigorously by practically all the streptococci from human and equine feces, but the streptococci

of man produce a markedly greater amount of acid than those from horse-dung. The mode for the human cultures lies between 3.6 and 4.0 per cent; that for the equine cultures between 1.6 and 2.0 per cent. The streptococci from cow-dung exhibit two distinct and clearly marked types, one forming about 2.0–2.5 per cent acidity, approaching the value of those from horse-dung, and the other group, of about the same numerical importance, forming no acid at all.

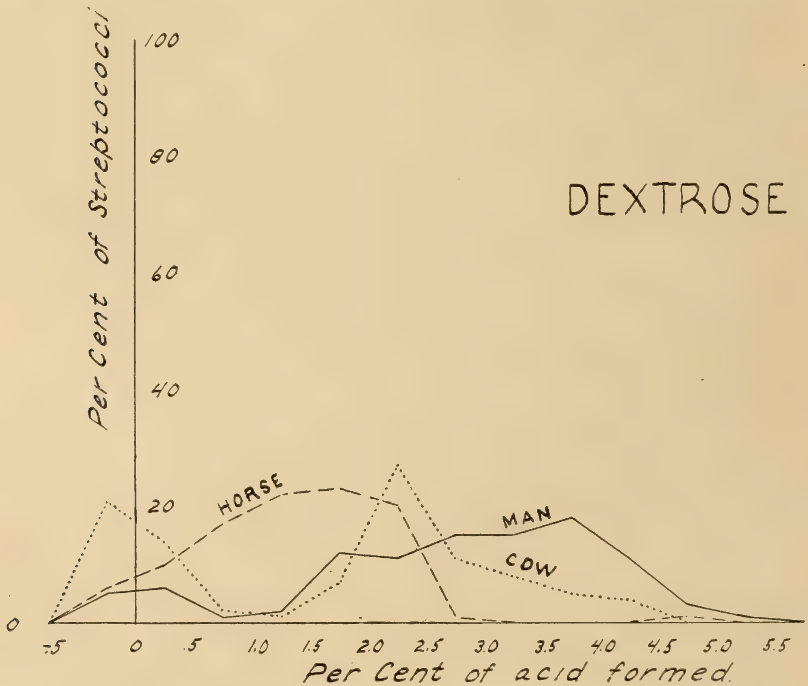

Chart 1.—Acid producing power of streptococci in dextrose broth.

In lactose the human and bovine cultures show two distinct types, one group yielding an acidity between 2.6 and 3.0 per cent, the other group producing no acid reaction at all. In the horse feces only the non-acid type is found.

Raffinose is not acted upon by any appreciable number of human or equine cultures. On the other hand, in the feces of the cow a small but clearly marked group appears, forming an acidity between 2.1 and 2.5 per cent.

Mannit is apparently not fermented by any important number of streptococci in the horse or cow but is acted upon by a small but definite group of the human strains.

TABLE 2.

STREPTOCOCCI GROUPED IN PER CENT ACIDITY CLASSES.

	−.5 0	.1 .5	.6 1.0	1.1 1.5	1.6 2.0	2.1 2.5	2.6 3.0	3.1 3.5	3.6 4.0	4.1 4.5	4.6 5.0	5.1 5.5
					Man (116)							
Dextrose......	6	7	1	2	14	13	17	17	21	13	4	1
Lactose.......	20	24	2	5	17	11	24	12	1	0	0	0
Raffinose.....	34	75	2	1	0	1	2	1	0	0	0	0
Mannit.......	47	36	6	4	12	0	1	0	0	0	0	0
					Horse (100)							
Dextrose......	6	10	17	22	23	20	1	0	0	0	1	0
Lactose.......	66	26	4	2	1	0	1	0	0	0	0	0
Raffinose	54	42	1	0	2	1	0	0	0	0	0	0
Mannit.......	62	36	0	1	1	0	0	0	0	0	0	0
					Cow (86)							
Dextrose......	18	12	2	1	6	23	9	7	4	4	0	0
Lactose.......	27	14	1	0	8	8	20	8	0	0	0	0
Raffinose.....	36	26	0	3	6	11	3	1	0	0	0	0
Mannit	54	27	2	3	0	0	0	0	0	0	0	0

These results are compared in a general way with those obtained by Houston (1905, 1906) and Andrewes and Horder (1906) in Table 3. In classifying our own results for this table we have considered all results under 0.5 per cent as negative. The various investigations are

TABLE 3.

COMPARATIVE RESULTS OBTAINED BY VARIOUS OBSERVERS IN REGARD TO FERMENTATIVE POWER OF INTESTINAL STREPTOCOCCI FROM VARIOUS SOURCES.

SOURCE OF FECES	OBSERVER	NUMBER OF CULTURES	PERCENTAGE OF POSITIVE RESULTS			
			Dextrose	Lactose	Raffinose	Mannit
Human..................	Houston	300	..	76	32	24
Human..................	Winslow and Palmer	116	89	62	6	28
Equine..................	Andrewes and Horder	13	..	0	0	0
Equine..................	Winslow and Palmer	100	84	8	4	2
Bovine..................	Houston	100	..	85	74	0
Bovine..................	Winslow and Palmer	86	65	52	28	6

concordant with the exception that raffinose-fermenters in both human and bovine feces were less frequent in our observations than in those of Houston. Our results also indicate a somewhat lower percentage of lactose-fermenters. All the investigations show that streptococci from the human intestine generally attack lactose while some strains ferment mannit; bovine strains attack lactose or raffinose; and equine strains dextrose only.

In determining what types of streptococci are present in each species of animal, the correlation of fermentative power must be taken into account, as well as the activity of the organisms in each particular sugar. In the horse the problem is a simple one for all the organisms studied belong to a single type, characterized by a moderate acid production in dextrose broth and failure to attack either of the other carbohydrates. This is clearly the Strept. equinus described by Andrewes and Horder. In the human and bovine feces, on the other hand, the problem is more complex, and can best be understood by grouping the organisms according to their relation to all the carbohydrates studied, which has been done in Table 4.

TABLE 4.

Intestinal Streptococci of Human, Equine, and Bovine Origin, Grouped According to Their Fermentative Reactions.

Carbohydrate Fermented	Name of Type	Percentage of Streptococci Found in		
		Man	Horse	Cow
None	....	9	15	18
Dextrose alone	S. equinus	23	73	27
Lactose alone	....	2	0	5
Dextrose and lactose	S. mitis	31	5	21
Dextrose and raffinose	....	0	3	3
Lactose and raffinose	....	0	0	12
Dextrose, lactose, and raffinose	S. salivarius	5	0	9
Dextrose, lactose, and mannit	S. fecalis	23	0	2
All four	....	0	1	3

One of the most striking results of this tabulation is the confirmation it affords of the reality of the type centers established by Andrewes and Horder. The strains which fermented no carbohydrates at all may be considered as weak forms which failed to establish themselves in any of the media. Aside from this class the only large groups of organisms were those named as type centers by the English observers, Strept. equinus, Strept. mitis, and Strept. fecalis. Strept. salivarius comes next; and the other combinations of characters are exhibited by so few strains that, with one exception, they may be considered as isolated variants from the commoner types. The single exception which may prove significant is the type fermenting lactose and raffinose but neither dextrose nor mannit, which made up 12 per cent of the fecal streptococci of the cow. If further study should confirm these results this type may deserve a specific name of its own.

Reviewing the results as arranged in Table 4 it appears that equine feces contain only one common type of streptococci, Strept. equinus, which attacks dextrose but cannot ferment the other carbohydrates.

In the streptococci of human origin on the other hand there are three common types. Of our 116 human cultures, 27 fermented dextrose only, 36 dextrose and lactose, and 27 dextrose, lactose, and

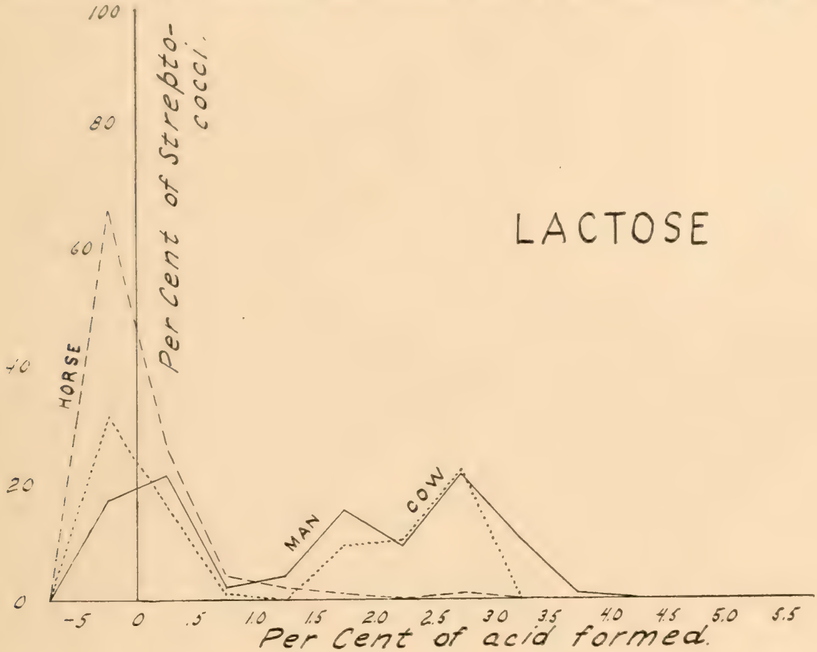

CHART 2.—Acid producing power of streptococci in lactose broth.

mannit. The first type is clearly allied to Strept. equinus; but, while these streptococci exhibit the same general qualitative relations to the sugars in the horse and man, their vigor of fermentative power, when measured quantitatively, is somewhat different. The equine strains as pointed out above produce an acidity in dextrose of about 2.0 per cent and the same is true of the bovine forms. On the other hand the human streptococci produce almost twice as much acid. There is apparently a distinct variety of Strept. equinus characteristic of the human intestine which may be recognized by its high fermentative power as measured in dextrose broth.

One interesting point about the non-lactose-fermenting streptococci of human feces, whether of the Strept. equinus type or of the group of weakened organisms which fermented neither sugar, was their association with diarrhea. We were especially anxious to see if characteristic streptococci were associated with this condition; and 8 of the 15 samples of human stools examined were more or less diarrheal in nature. Of 31 streptococci from normal stools, only 4 failed to ferment lactose; while of 85 strains from diarrheal stools

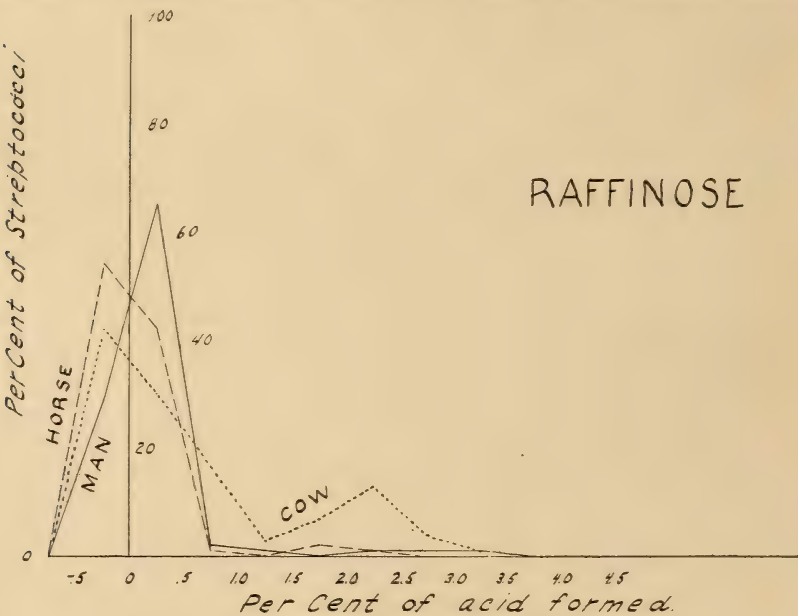

CHART 3.—Acid producing power of streptococci in raffinose broth.

36 formed no acid from that sugar. Twenty-seven of the 36 fermented dextrose vigorously and 9 were weak forms which produced no acid in any medium. The 27 strains which fermented dextrose only form an interesting group differing, as noted above, in the amount of acid produced, from the type Strept. equinus. Twenty-four of these 27 organisms came from a single individual, appearing in three different samples of diarrheal stools. Altogether 47 strains of streptococci were isolated from these three stools; 24 of them were of the Strept. equinus type and 19 of the 24 formed more than 3 per cent acid in dextrose. The other three human strains of Strept.

equinus were from the diarrheal stools of other persons; and the connection may prove to be something more than a personal idiosyncrasy.

The second type of streptococci found in human feces, the first in point of abundance, was Strept. mitis, which ferments dextrose and lactose but not raffinose and mannit. This organism was the commonest form in human feces and second in abundance in cow-dung.

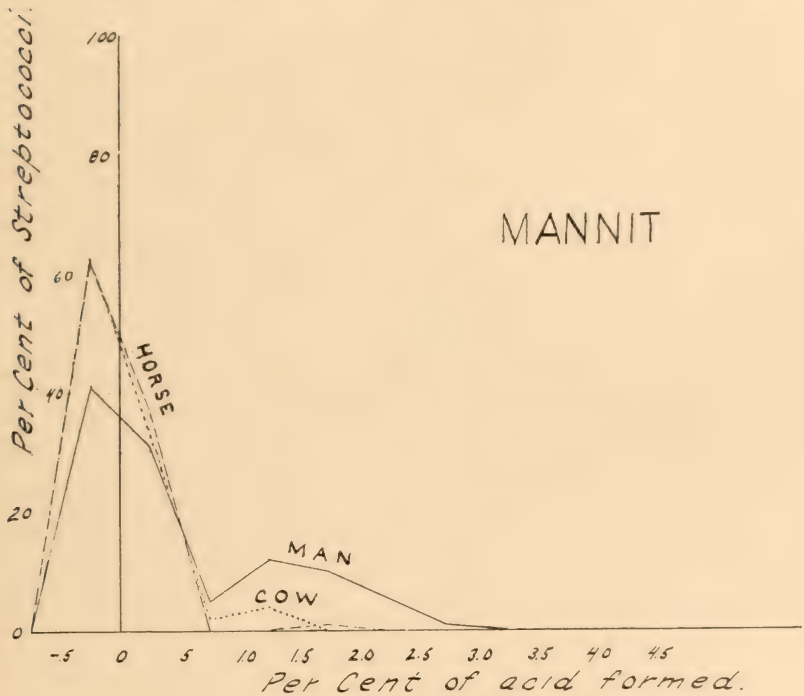

CHART 4.—Acid producing power of streptococci in mannit broth.

Finally 23 per cent of the human strains belonged to the type of Strept. fecalis, characterized by fermentation of dextrose, lactose, and mannit. This form was not found at all in the feces of the horse and but twice in that of the cow, these results corresponding closely with those obtained by Houston.

The intestinal flora of the cow appears to be more complex than either of the others. Strept. equinus and Strept. mitis are the commonest types but two other forms were also present in considerable

numbers. Nine per cent of the strains belonged to the type of Strept. salivarius, characterized by the fermentation of dextrose, lactose, and

TABLE 5.
Streptococci Isolated from Human Feces.

No.	Sample No.	Percentage of Acid Formed				No.	Sample No.	Percentage of Acid Formed			
		Dextrose	Lactose	Raffinose	Mannit			Dextrose	Lactose	Raffinose	Mannit
1	25	4.4	3.0	.1	.1	59	78	3.3	0	−.2	−.1
2	26	3.1	2.7	.4	1.7	60	78	2.9	0	+.2	−.1
3	26	3.9	3.0	.2	1.6	61	78	1.1	.2	+.2	−.2
4	26	3.6	2.8	0	.2	62	78	3.5	0	.1	0
5	27	3.4	3.0	.2	1.5	63	78	2.9	0	−.1	−.3
6	27	3.0	2.8	.2	.1	64	78	1.6	1.1	−.0	−.2
7	27	3.6	2.7	.3	.3	65	78	1.7	1.8	+.2	−.2
8	27	3.6	2.7	.3	.2	66	78	2.0	1.6	−.0	−.2
9	27	4.4	3.1	.1	.3	67	78	.1	0	−.2	−.1
10	27	3.8	2.5	.1	.9	68	78	3.2	0	−.0	−.2
11	27	3.0	3.1	.2	.4	69	78	4.4	2.7	+.2	−.1
12	27	4.1	2.9	.2	.3	70	78	.1	.1	−.0	−.2
13	27	3.8	3.0	.4	.2	71	78	0	.1	+.1	−.1
14	27	3.0	2.7	.1	.4	72	78	−.1	.1	+.1	−.1
15	27	3.3	2.8	0	1.7	73	78	2.6	0	0	1.9
16	28	3.9	3.2	0	.1	74	78	3.2	0	−.1	.8
17	28	4.3	2.8	.4	.1	75	78	3.2	0	.1	−.1
18	28	3.8	3.0	.1	1.3	76	78	3.0	0	.1	.3
19	49	4.3	2.4	.1	1.4	77	79	4.1	2.3	.3	2.6
20	49	4.0	2.8	.3	1.4	78	79	1.9	1.6	.2	1.6
21	49	4.1	2.9	.3	1.1	79	80	2.1	1.8	.2	1.6
22	49	3.9	2.7	.3	1.3	80	80	2.0	1.7	.4	1.7
23	49	4.0	3.1	0	1.2	81	80	2.0	1.7	.2	1.6
24	49	3.8	3.1	.3	1.2	82	80	2.1	1.6	.4	1.7
25	50	2.7	3.7	.9	0	83	80	2.2	.1	.3	1.5
26	50	2.3	2.8	1.4	.1	84	80	1.9	1.6	.2	1.6
27	50	2.6	3.5	1.0	0	85	80	1.9	1.7	.3	1.5
28	65	3.8	3.3	.2	.2	86	80	2.1	1.6	.3	1.7
29	65	.5	.1	.2	0	87	80	1.8	1.7	.4	1.5
30	65	4.6	3.1	.1	−.1	88	80	1.9	1.6	.2	1.5
31	65	4.2	.2	.1	.3	89	80	1.9	1.4	.4	1.8
32	65	4.5	2.3	3.2	.1	90	80	1.5	1.6	.2	1.5
33	65	.3	.2	.1	.1	91	81	2.3	2.7	.1	−.2
34	65	4.6	2.4	.2	.2	92	81	2.3	.1	.1	.1
35	65	3.6	0	.1	.3	93	81	1.7	3.0	.1	−.1
36	65	4.3	2.4	3.0	.1	94	81	−.1	2.9	0	−.1
37	65	2.7	0	.2	.1	95	81	2.5	2.8	2.6	−.3
38	65	.1	.1	.2	.2	96	81	.7	2.3	.1	−.1
39	65	2.9	0	.1	.3	97	81	2.1	1.4	−.1	−.2
40	65	4.0	.1	0		98	81	2.3	2.8	2.5	−.2
41	65	3.7	.1	.1		99	81	2.2	3.1	0	0
42	65	0	.2	0		100	81	1.6	3.1	0	.1
43	65	3.4	.1	0	0	101	88	3.4	.2	.1	.8
44	65	3.5	.1	0	−.1	102	88	3.2	1.9	0	1.1
45	65	3.5	0	−.1	0	103	88	4.5	2.1	.2	.8
46	65	3.9	0	0	0	104	88	4.8	2.4	0	−.1
47	65	3.4	0	0	0	105	88	3.6	.4	.2	.8
48	65	3.8	.1	0	0	106	88	2.9	2.2	.1	.3
49	65	3.4	.1	0	0	107	88	3.2	.5	.3	.6
50	65	.1	0	0	0	108	88	2.8	1.0	.1	.1
51	66	1.8	2.0	−.1	.1	109	89	.2	1.2	0	0
52	66	3.8	.3	.4	−.1	110	89	2.4	1.6	.2	−.1
53	66	4.9	3.3	.2	.1	111	89	2.7	−.1	.1	−.1
54	66	3.8	.3	.2	.1	112	89	0	−.2	.1	0
55	66	3.7	.1	.1	0	113	89	2.7	1.7	0	.4
56	66	5.4	3.2	.2	0	114	89	2.5	1.4	.1	.1
57	74	0	.1	−.0	−.1	115	89	2.7	.7	0	−.1
58	74	4.1	2.4	+.2	−.1	116	89	3.1	−.1	.1	−.1

raffinose. Twelve per cent of the cultures were of a new type, not apparently described hitherto, having the peculiar property of attack-

ing lactose and raffinose but not dextrose. This is highly unusual since dextrose as the simplest sugar is almost always fermented before any other carbohydrate. We have hesitated however to give a name to this type while it is characterized by only 10 cultures.

TABLE 6.

STREPTOCOCCI ISOLATED FROM HORSE-DUNG.

No.	Sample No.	Percentage of Acid Formed				No.	Sample No.	Percentage of Acid Formed			
		Dextrose	Lactose	Raffinose	Mannit			Dextrose	Lactose	Raffinose	Mannit
1	32	4.0	1.1	.8	1.9	51	46	.9	0	.1	0
2	35	1.4	1.3	.2	.3	52	46	0	.6	0	−.1
3	36	2.0	0	0	.1	53	46	1.9	.2	0	−.2
4	36	1.3	.2	0	0	54	46	1.9	−.2	0	0
5	36	1.7	.1	0	.1	55	47	2.2	2.7	.1	.1
6	36	2.1	0	.3	0	56	47	1.3	0	.1	0
7	36	.4	0	.2	0	57	47	1.9	0	0	0
8	36	2.1	0	.3	.1	58	47	2.1	−.1	0	−.1
9	36	1.9	0	0	0	59	47	1.6	−.1	0	0
10	36	.8	.6	.1	.2	60	47	1.6	.1	.2	0
11	37	2.6	0	2.8	.2	61	47	1.7	0	.1	−.1
12	37	1.1	.2	0	0	62	47	1.2	−.1	0	−.1
13	37	1.8	0	0	0	63	47	.2	−.1	−.1	0
14	37	1.0	0	.5	.3	64	47	2.4	−.1	0	−.1
15	37	2.3	0	.2	.1	65	47	2.3	.1	.2	0
16	37	2.3	0	.3	.1	66	47	2.5	0	0	1.2
17	37	1.0	0	.3	0	67	47	2.4	.1	0	0
18	37	.6	0	0	0	68	48	.2	−.4	.2	−.1
19	37	2.3	0	0	0	69	48	1.1	.3	−.1	0
20	37	2.5	0	0	.1	70	48	.1	0	.1	−.1
21	37	1.4	0	0	.1	71	48	1.5	−.2	0	−.3
22	37	1.5	0	0	0	72	48	2.2	−.3	0	−.2
23	37	1.6	0	0	.1	73	48	1.9	0	.1	−.1
24	37	1.8	1.6	0	0	74	48	2.0	0	−.1	−.2
25	38	.2	.2	0	0	75	48	1.5	.2	0	−.1
26	38	0	0	0	0	76	48	1.9	0	0	0
27	38	2.1	0	0	.3	77	48	1.8	−.1	0	0
28	38	1.4	0	.5	.1	78	48	−.1	0	0	−.2
29	38	1.5	0	.3	0	79	64	1.1	.1	0	0
30	38	1.9	0	.3	.1	80	64	1.3	0	0	.1
31	38	2.5	0	.5	0	81	82	1.0	.1	.1	.1
32	38	1.3	0	.2	.3	82	82	.8	0	0	−.1
33	38	2.0	.3	1.7	.1	83	82	1.1	0	.1	.1
34	38	2.3	0	0	0	84	82	.9	.1	.4	.1
35	38	.2	0	0	0	85	82	.8	.1	.1	0
36	38	1.9	0	0	0	86	82	.9	.1	.1	0
37	38	2.5	0	0	0	87	82	.8	0	.1	.1
38	38	2.1	0	.3	.1	88	82	1.4	.8	.2	.1
39	39	.3	.2	.3	.2	89	82	1.8	.6	.2	.1
40	39	.4	0	0	.2	90	82	1.2	−.1	.1	−.1
41	39	2.1	0	0	.1	91	82	.9	.1	0	.1
42	39	2.3	.1	0	0	92	82	1.1	0	.2	.1
43	39	1.8	.1	0	.2	93	82	.9	.1	.1	−.1
44	39	0	0	.3	.2	94	82	.8	.1	.1	0
45	39	0	0	.3	.1	95	82	.2	−.1	0	.1
46	39	0	0	.4	0	96	82	.8	.1	0	.1
47	39	1.3	0	.2	0	97	82	1.2	.1	0	.1
48	39	1.6	0	0	.1	98	82	1.1	.1	−.1	0
49	39	.2	0	0	0	99	87	.9	0	−.1	−.1
50	39	2.0	.3	2.0	0	100	87	1.0	0	0	0

CONCLUSIONS.

The general result of our investigations has been to confirm and extend the conclusions of the English bacteriologists. We have found, as Andrewes and Horder concluded from their analysis of

Houston's descriptions, that the chief types of streptococci in the normal human intestine are Strept. mitis, fermenting dextrose and lactose, and Strept. fecalis, fermenting dextrose, lactose, and mannit. In addition we would call attention to the presence of a peculiarly vigorous type of Strept. equinus fermenting dextrose only.

TABLE 7.
STREPTOCOCCI ISOLATED FROM COW-DUNG.

No.	Sample No.	Percentage of Acid Formed				No.	Sample No.	Percentage of Acid Formed			
		Dextrose	Lactose	Raffinose	Mannit			Dextrose	Lactose	Raffinose	Mannit
1	41	2.1	2.0	0	0	44	69	3.0	.2	0	0
2	41	.6	0	0	0	45	69	0	.1	.1	.1
3	43	2.7	2.5	.1	0	46	69	.1	0	0	.1
4	44	3.4	3.4	1.2	.1	47	69	0	.2	0	0
5	44	2.9	2.2	0	0	48	69	2.0	2.0	0	0
6	44	2.1	2.1	0	.1	49	69	2.5	.2	.1	0
7	44	2.3	2.1	0	0	50	69	.2	.1	0	0
8	44	.2	3.0	2.6	0	51	69	2.5	.1	−.1	.1
9	53	2.3	2.4	2.0	0	52	69	0	2.0	0	0
10	53	0	3.1	2.0	0	53	69	2.5	1.7	−.1	0
11	53	4.1	2.9	0	.8	54	69	.1	.3	.1	0
12	54	2.2	2.6	1.7	0	55	69	.1	.1	0	0
13	54	2.1	2.6	2.0	.1	56	69	2.2	.1	−.1	0
14	54	−.1	−.2	−.1	−.1	57	70	2.5	−.1	.1	.1
15	54	−.1	2.9	−.1	0	58	71	2.3	0	.2	.1
16	54	0	3.1	2.0	−.1	59	71	2.5	0	0	−.1
17	54	−.2	3.0	2.3	1	60	71	2.4	−.1	.1	0
18	54	−.1	2.8	2.3	0	61	71	.1	−.1	.2	−.1
19	54	−.1	2.9	2.4	.1	62	71	2.5	−.1	.1	0
20	54	3.1	2.7	−.1	.1	63	71	2.8	0	.1	0
21	54	−.2	2.8	−.1	0	64	71	2.0	0	.1	−.1
22	54	3.3	3.0	.1	−.2	65	72	3.0	1.6	.2	.1
23	56	2.1	−.1	2.1	0	66	72	.3	−.1	0	−.1
24	56	3.7	2.6	−.1	1.4	67	72	3.3	−.1	0	.2
25	56	2.4	2.5	2.3	−.1	68	72	2.6	2.0	.1	−.1
26	56	0	2.9	2.3	.2	69	72	2.5	−.1	.1	.1
27	56	−.1	3.1	2.4	.1	70	72	2.4	.1	2.6	0
28	56	−.1	2.5	2.3	.1	71	72	2.2	−.2	.1	0
29	56	4.2	3.1	2.0	1.3	72	72	0	0	.0	0
30	56	0	2.9	2.3	0	73	72	2.2	0	.1	.2
31	56	3.5	2.9	2.5	−.1	74	72	1.9	−.1	.1	.1
32	59	3.5	2.9	0	.1	75	75	4.1	2.7	.2	0
33	61	3.5	2.9	0	0	76	77	3.7	.6	.1	−.1
34	61	4.2	3.1	0	.1	77	86	1.0	0	.1	.1
35	61	3.9	3.2	3.5	0	78	91	−.2	.1	.1	.1
36	61	3.9	3.5	0	0	79	91	.1	0	.2	.2
37	67	2.0	.2	0	0	80	92	2.3	1.6	1.4	1.3
38	67	1.1	.2	0	0	81	92	2.2	1.9	1.5	.9
39	68	.4	0	.1	0	82	92	.1	0	.1	.2
40	68	2.8	.2	−.1	0	83	96	.2	0	.2	0
41	68	2.6	.1	0	0	84	96	1.7	1.2	2.3	.1
42	68	0	2.3	−.1	0	85	98	2.8	2.6	3.0	0
43	69	2.0	0	0	.1	86	97	.3	0	0	−.1

We have found, as Andrewes and Horder did, that the characteristic streptococcus of the horse is the non-lactose-fermenting Strept. equinus; and this appears to be the only form typically present. Seventy-three per cent of all our equine strains belonged clearly to this type.

In the feces of the cow, on the other hand, streptococci which fail

to ferment lactose are relatively less common, as Houston showed. Strept. equinus is present; but so are Strept. mitis and Strept. salivarius (fermenting dextrose, lactose, and raffinose); and we have found in small numbers a peculiar new type fermenting lactose and raffinose but not dextrose. Strept. fecalis, as in the horse, is absent.

From the standpoint of the water bacteriologist several conclusions may be drawn. In the first place, it appears that pollution with road washings may be distinguished from wastes of other sorts by a study of the streptococci present. Since most of the pollution in street washings comes from horse-dung, and since lactose-fermenting streptococci are comparatively rare in such deposits, a test for these organisms should have distinct value. Such a test might easily be made by inoculating tubes of lactose broth, incubating for several days, and then plating on lactose agar.

The distinction between human and bovine pollution is also promising. There are three points of difference which seem to deserve investigation. First the presence of streptococci forming over 3.5 per cent of acid in dextrose broth would seem in general to be characteristic of human stools. Second, raffinose-fermenting forms (Strept. salivarius) appear to be more abundant in bovine than in human feces. Third, and of most importance, mannit-fermenting streptococci (Strept. fecalis), which make up about one-quarter of the human streptococci, are very rare in the feces of the horse and cow. In this respect Houston's results and our own are in complete agreement; and the use of mannit broth as a differential test for streptococci of human origin would seem sufficiently promising to warrant further study.

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AN INVESTIGATION OF THE EXTENT OF THE BACTERIAL POLLUTION OF THE ATMOSPHERE BY MOUTH SPRAY.*

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INTRODUCTION.

With the progress of exact knowledge sanitarians have been led to place less and less emphasis upon the part played by the atmosphere in the spread of zymotic disease. Bacteriological studies have shown that quietly expired air is germ free and epidemiological investigations indicate that little place is left for aerial transfer of disease in any form (Chapin, 1908). Even sewer air has been shown by recent work to be on the whole remarkably free from pollution with sewage bacteria.

On the other hand, Horrocks and Andrewes in England have demonstrated that a local pollution of sewer air does occur in the presence of mechanical splashing, taking the form of a fine spray of sewage, temporarily suspended in the air. Similarly, the work of Flügge and his pupils has shown that in sneezing, coughing, and loud speaking a spray is thrown out which contaminates the air for a considerable distance with bacteria from the mouth. It has been generally assumed that this mouth spray may be an important factor in the spread of tuberculosis, and other diseases affecting the respiratory tract.

The real importance of the mouth spray, as of any other vehicle of disease, can be properly determined only by quantitative investigations. The day of qualitative sanitation is fast passing. Not, "Is such a thing dangerous?" but, "How dangerous is it?" is the question we ought to ask. Applying this criterion to the bacterial content of sewer gas one of the writers has been led to the conclusion that on the whole the danger of infection from that medium is very slight (Winslow, 1909). In the present investigation the attempt has been made to apply similar quantitative standards to the mouth spray,

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and to determine whether the extent of air pollution with mouth bacteria under normal conditions is sufficient to be measured bacteriologically and to be estimated as a practical factor in the spread of zymotic disease.

PREVIOUS INVESTIGATIONS ON THE DISCHARGE OF BACTERIA IN MOUTH SPRAY.

It is particularly to Flügge and his pupils that we owe the most careful studies of the possibilities of aerial transmission of disease germs, either in the form of dust or in the form of fine spray (Flügge, 1897*a* and 1897*b*). He was the first to lay special stress upon the danger from the spray produced in coughing in the case of tuberculosis (Flügge, 1899), and to point out how much more important this danger is than the possibility that the disease may spread through the blowing about of dried sputum which before this time had been accepted on the basis of Cornet's classic work (Cornet, 1889) as the principal mode of disseminating the disease.

TABLE 1.

DISTRIBUTION OF BACTERIA BY MODERATELY LOUD SPEAKING.
Laschtschenko (1899).
Room, 90 cu.m. Speaking for 1 hour.

Distance of plate from speaker. Meters	0.5	0.75	1.0	1.25	1.5	1.75
Total plates exposed	1	3	1	2	3	2
Negative plates	0	0	0	0	0	0
Mean number B. prodigiosus per plate	450	32	10	6	4	8

TABLE 2.

DISTRIBUTION OF BACTERIA BY LOUD SPEAKING (ONE HOUR).
Laschtschenko (1899).

	Mean Number B. prodigiosus per Plate					
Distance. Meters	1	2	3	4	5	6
Small room (50 cu.m.)	1	3	2	0	1	1
Large room (90 cu.m.)	5	10	..	3	..	..

The first important experiments upon mouth spray were carried out by Laschtschenko, and upon them Flügge's earlier papers were largely based, altho the full results were not published until two years later (Laschtschenko, 1899). The general method employed in these experiments consisted in inoculating the mouth with a heavy suspension of B. prodigiosus in normal salt solution and then causing the subject to cough, sneeze, or speak loudly for a specified length of time. Agar plates were exposed at various distances to receive any bacteria which might settle upon them. Some of the experiments were made in a glass chamber of 3.2 cubic meters capacity provided with an opening for the mouth of the investigator; others were carried out in rooms of 50 and 90 cubic meters capacity.

Quiet speaking, in the glass chamber, showed a few colonies; in the larger room, none. The results for moderately loud speaking and for loud speaking we have tabulated for convenient reference in Tables 1 and 2.

A general summary of all the loud and moderately loud speaking experiments grouped together is given in Table 3.

Coughing and sneezing of course gave higher results which need not be considered in detail. Sneezing distributed the infection for nine meters.

The most significant of Laschtschenko's experiments were carried out with tuberculous patients. The coughing of such a patient while sitting for an hour in the glass chamber, to which reference has been made, distributed virulent tubercle bacilli, as evidenced by animal tests made with normal salt solution exposed in the chamber during the test. The actual extent of the air pollution was however slight, as shown by drawing off the air of the chamber, filtering, and injecting into guinea-pigs. When less than 2 cu.m. of air per hour were examined, no positive results were obtained; but out of five tests covering a period of five hours each, and involving the filtering of 10 cu.m. of air in each case, two tests showed virulent tubercle bacilli.

Hübener (1898) carried out a series of similar experiments with a view to the danger of infecting patients upon the operating table from the mouth. His method consisted in exposing four agar plates about 50 cm. in front of and below the face of a subject who had infected his mouth with B. prodigiosus. In 11 experiments in which the subject counted aloud for 10 minutes the number of colonies on the four plates

TABLE 3.

GENERAL SUMMARY OF LASCHTSCHENKO'S EXPERIMENTS.

	90 cu.m. Room	50 cu.m. Room
Total plates	24	12
Mean number B. prodigiosus per plate	25	1.5
Per cent of negative plates	8	33

varied from 101 to 1,507 and averaged 458; two tests with three or four coughs gave 223 and 265 colonies and in two tests a single sneeze gave an uncountable number.

Along similar lines, Hamilton (1905) has shown that streptococci are expelled from the mouth in coughing, speaking, and even forcible breathing, and has emphasized the danger of surgical infection from this source.

V. Weismayr (1898) obtained somewhat different results which indicated a more restricted distribution of mouth spray than that reported by other observers. He found B. prodigiosus on plates directly in front of a coughing subject in quiet air up to a distance of 4 meters, but not beyond and not out of the zone directly in front of the mouth. When the air of the room was agitated, however, he found small numbers of bacilli 1–2 meters behind and at the side of the subject. In speaking experiments infection could not be traced at distances over 1 meter. Slides exposed before the mouths of coughing consumptives showed tubercle bacilli only rarely.

The most exhaustive of all the experimental studies of the spread of B. prodigiosus in mouth spray was perhaps that carried out by Koeniger (1900). He followed in the main the methods of Laschtschenko, making in all 18 speaking experiments in two rooms, one of 97, the other of 440 cu.m. capacity. His main results have been summarized in Table 4.

Koeniger observed also the amount of infected spray discharged by enunciating various letters and showed that consonants like *p* and *t* caused the greatest pollution. The amount of spray of course increased with loudness and sharpness of speech but, as indicated in the table below, whispering, with its clear enunciation, gave higher results than gentle speech in an ordinary tone.

TABLE 4.

DISTRIBUTION OF BACTERIA IN MOUTH SPRAY.
(Koeniger, 1900.)
Speaking, generally for 15 minutes.

	Tone of Voice				
	Sharp	Loud	Moderately Loud	Soft	Whisper
Total plates	30	101	143	23	24
Mean number B. prodigiosus per plate	14.5	7.8	2.1	1.4	5.3
Total negative plates	0	9	91	16	10
Per cent negative plates	0	8.9	63.6	69.6	41.7

Koeniger found B. prodigiosus on his plates in all parts of the room, at extreme distances of 12.4 meters in front of the speaker and at one side of and behind him. In this respect his results differ from those of v. Weismayr. On the other hand, he made important observations on the length of time for which the bacilli would remain in the air which contradict Flügge's conclusion, based on experiments with atomizer spray, that bacteria from the mouth might remain suspended in the air for 4 or 5 hrs. Koeniger's general results are brought together in the table below. B. prodigiosus was never found in quiet air an hour after the speaking had ceased, tho when the air of the room was agitated by moving about and opening and shutting doors, positive results were obtained after an hour and a half.

TABLE 5.

SETTLING OUT OF MOUTH SPRAY IN QUIET AIR.
(Koeniger, 1900.)

Interval between Speaking and Exposure of Plates. Minutes	Percentage of Colonies Found, Taking Number on Plates Exposed during Speaking as 100
0	95
10	38
20	9.8
30	5.5
45	2.7
60	0.7
90	0.0
105	9.6*
120	0.0
150	0.0

* Air agitated.

Experiments with B. mycoides made in similar fashion showed a much more limited distribution of mouth spray than was observed in the case of B. prodigiosus. Out of 105 plates exposed during 15 minutes' speaking only 23 showed colonies of the specific germ. The average number of colonies per plate was 7 and the maximum distance 3 meters. All plates exposed 10 minutes after speaking were sterile.

Gordon in England was the next to take up this work and his investigations confirmed in all respects the work of Laschtschenko and Koeniger (Gordon, 1904). Repeating the B. prodigiosus experiments he traced aerial infection for 12.2 meters in front of the subject and for 3.7 meters behind him. We have summarized these particular experiments in Table 6 below. Gordon's most important contribution to the whole subject lay in the suggestion that the mouth streptococcus might be used as a normal

index of mouth pollution without the use of artificial B. prodigiosus inoculations; but this aspect of his work will be considered farther on.

Mention may perhaps be made here of experiments by Flügge (1897), Buchner, Megele, and Rapp (1899), and Hutchison (1901), which have shown, as might be expected, that bacteria artificially sprayed into the air can be transported by air currents of low velocity for considerable distances. Buchner, Megele, and Rapp found a limiting velocity of 1 mm. per second for B. prodigiosus and 1.3–1.8 mm. per second for yeast cells 3–10 microns in diameter. Little can be learned from such experiments as to the behavior of the mouth spray itself which may be in a much grosser state of division.

So far reference has been made for the most part only to experiments in which B. prodigiosus was used as an indicator of the distribution of bacteria in the mouth spray. Another important series of investigations has dealt more directly with the practical

TABLE 6.

Distribution of Bacteria in Mouth Spray.

(Gordon, 1904.)

Generally, loud speaking for 1 hour.

Total plates exposed	30
Mean number B. prodigiosus per plate	0.7*
Total negative plates	14
Per cent negative plates	47

* Number on four positive plates not stated.

question of the spread of tuberculosis by observations of the behavior of the tubercle bacillus itself. Laschtschenko's work along this line has already been discussed. At about the same time Englemann (1898) and Heymann (1899) showed that tubercle bacilli could be detected on plates of various sorts exposed within 0.5–1 meter of coughing consumptives; and Heymann succeeded in infecting 6 out of 25 guinea-pigs exposed 20–45 cm. in front of coughing consumptives, with heads fixed toward the patients.

Fränkel (1899) found that out of 219 masks worn by tuberculous patients 52 showed macroscopic evidence of contamination with sputum; in 26 of the latter tubercle bacilli could be demonstrated by staining. Fourteen patients wore the masks in these experiments. Two yielded positive results almost always, one frequently, 3 once each, and 8 never showed tubercle bacilli on the masks.

In a later investigation, Heymann (1901) shut up a coughing consumptive patient in a small chamber for an hour or two. At the close of this period the patient left the chamber and broth plates were exposed, after the chamber had remained quiet, for varying periods, 15, 30, or 90 minutes. Of 24 guinea-pigs inoculated with broth thus exposed, two which had received broth exposed 30 minutes after the end of the coughing developed tuberculosis. In a second series of tests the air in the chamber was drawn off and washed, and the sediment in the washings centrifuged and injected. Of 14 guinea-pigs injected with the sediment from 7 cu.m. of air drawn off while the coughing was in progress only two became infected; of 22 guinea-pigs injected with the sediment from 11 cu.m. of air drawn off at periods of 15–60 minutes after the coughing stopped, all remained sound. Many more recent observers have shown that tubercle bacilli may be found on plates exposed before coughing consumptives. The most exhaustive of these investigations is perhaps that of Ziesché (1907). He arranged a

vertical plate having an area of 324 square centimenters at a distance of 40–80 cm. from the mouth of phthisical patients and counted under the microscope the number of tubercle bacilli discharged in half an hour. Of the patients examined only once 12.5 per cent gave positive results; of those frequently examined, 78.9 per cent. Altogether 29 out of 62 tests were positive. Of the 29 positive plates, 3 showed less than 10 bacilli, 15 from 10 to 100, 7 from 100 to 500, 2 from 500 to 1,000, one 1,445, and one 20,174 bacilli.

Similar observations have been made in the case of leprosy. Schäffer (1898) found leprosy bacilli on glass plates exposed before the mouth of a leper after 10 minutes' reading. Plates near the mouth showed many thousands of bacilli and infection could be traced to a distance of 1.5 meters. These experiments were repeated and confirmed by Hübener (1898) in a paper to which reference has been made above.

It is sufficiently clear from a consideration of all these investigations that bacteria may indeed be discharged from the mouth in such a way as to spread disease. On the other hand it seems equally clear from the tuberculosis experiments that the practical danger is confined to a local discharge of spray rather close to the mouth of the patient. Rightly interpreted there is no basis in any of this work for the conclusion that disease is spread broadcast through the atmosphere. Professor Flügge's own treatment of the matter is judicial and conservative. He lays stress only on coughing, not on speaking, as a possible danger. He points out that in experiments carried out by his pupils tubercle bacilli were abundant only within 0.5 meter of the coughing patient and that beyond 1.5 meters their number was so small as to make the chance of infection practically nil. He notes that only certain phthisical patients discharge infected spray when they cough and that others do so only at certain periods of the disease, and only at certain times of day. In one of his first papers (Flügge, 1897*b*), he says: "Altogether it appears that under natural conditions the infection resulting from sputum spray is not so significant as one might think at first sight." In a later paper (Flügge, 1901) the following rational rule is laid down: "During strong paroxysms of coughing, the consumptive should keep at arm's length from his companions and should hold a handkerchief before his mouth. In workrooms, offices, and such places the space between the heads of the workers should be at least 1 meter."

No exception can be taken to such conclusions as these. Unfortunately, however, others have not been so conservative. The B. prodigiosus experiments particularly have lent themselves to mis-

leading interpretations. It is common to see statements to the effect that bacteria are discharged from the mouth in coughing, sneezing, and even loud speaking to distances of 12 meters and in all directions behind as well as in front of the subject; and from such unqualified statements the inference is implicitly drawn that a general infection of the atmosphere takes place over a wide radius and that there is real danger of infection from breathing the air in the neighborhood of a consumptive. Fränkel (1899) early took up an extreme position, speaking of spread through the air as the "most frequent cause of tuberculosis," and recommended the wearing of masks by tuberculosis patients discharging bacilli freely. This was ten years ago, it is true; but Tendeloo (1908) at the last Tuberculosis Congress said: "It has been proved by numerous experiments (Cornet, Flügge, etc.), that bacteria floating in the air, either in dry dust particles, or suspended in minute fluid particles, may be inhaled into the bronchi, bronchioli, and air-vesicles, where they fall down just like inhaled dust particles;" Pannwitz (1908) said: "If they contain a coughing tuberculous patient, inclosed spaces, such as dwelling-houses, workshops, counting-rooms, public halls, railway coaches, and steamer cabins, are speedily converted into tuberculosis-inhalatoriums in which healthy persons may infect themselves;" and Bernheim (1908) recommended the wholesale disinfection of air on the ground that "there is an intimate relation between tuberculosis and the microbes of the air. The more highly the atmosphere is charged with bacteria the greater is the number of consumptives living in that unhealthy environment."

As a matter of fact it can be shown that even the B. prodigiosus experiments do not indicate any general atmospheric infection in the proper meaning of the term; and studies of the discharge of tubercle bacilli and of normal mouth bacteria show that the spread of these organisms is insignificant in comparison with the discharge of B. prodigiosus in culture media with which the mouth has been artificially infected.

REPETITION OF EUROPEAN EXPERIMENTS ON AIR POLLUTION BY MOUTH SPRAY USING B. PRODIGIOSUS AS A TEST ORGANISM.

The first object of our own experiments was to extend and place on a quantitative basis the work of Flügge's pupils on air pollution

by mouth spray. It was necessary therefore to follow their procedure rather closely, using B. prodigiosus as a test organism and detecting it by the simple exposure of plates, in order to be sure that the conditions of their work were fairly duplicated. At the same time we examined measured volumes of air and determined the actual number of specific germs present by methods discussed in the succeeding section.

Three methods were employed in our experiments for inoculating the mouth with B. prodigiosus. The first was to pour water on an agar slant culture, shake so as to get a suspension, rinse out the mouth with the suspension for a minute, and reject the surplus water. The second was to transfer the organisms directly from the culture to the mouth by means of a platinum loop. The third was to transfer the bacteria on a loop to a small quantity of water (1 or 2 c.c.) which could be entirely retained in the mouth. The last method proved most satisfactory. Analyses of the saliva at the close of the experiments generally showed that B. prodigiosus was present in 10^{-6} c.c.

In the first 10 experiments the subject read from a book in a loud tone and at a fairly rapid rate. Beginning with Experiment 11 and continuing through the series the subject repeated from memory verses in English and German, in a loud tone and with exaggerated enunciation. The discarding of the book appeared to facilitate the spread of bacteria.

The tests were carried out in three laboratories of the Institute of Technology. Two of them are about 6×8 meters in area and 3.7 meters high; the third is 11×8 meters and 3.7 high. In the qualitative work ordinary plates were exposed in different parts of the rooms at varying heights and at varying distances from the speaker. The speaking usually lasted for 15 minutes and the plates were exposed during this time and for a varying period, generally three-quarters of an hour afterward. The speaking was done by four different subjects during the course of the experiments.

The general results obtained by the simple exposure of Petri plates are indicated in Table 7. The speaking was continued for 15 minutes in each case except in Experiments 14 (7 minutes), 15 (13 minutes), and 21 and 23 (10 minutes).

It will be noticed that the substitution of speaking for reading,

TABLE 7.

B. Prodigiosus on Plates Exposed in Room Polluted by Mouth Spray.

Experiment	Period of Exposure Minutes	Number of Plates	Total Colonies	Mean per Plate	Negative Plates Total Number	Negative Plates Percentage
7	15	11	1	.09	10	91
8	15	12	2	.16	11	92
9	15	18	2	.11	16	89
10	15	21	4	.19	17	81
11	75	25	22	.88	14	56
12	15–75	42	3	.07	39	93
13	75	24	22	.92	10	42
14	..	25	9	.36	18	72
15	73	25	20	.80	16	64
16	135	13	34	2.6	3	23
17	..	11	68	6.2	3	27
18	45	10	134	13.4	0	0
19	60	12	88	7.3	0	0
20	60	14	75	5.4	0	0
21	60	14	142	10.1	0	0
22	75	12	56	4.7	4	33
23	25–70	36	98	2.7	8	22
Totals and averages		325	780	2.4	169	52

at and after Experiment 11, caused an increase in positive results and that as the later experiments progressed the number of bacteria on the plates continued to increase, perhaps as a result of more thorough inoculation of the mouth or more vigorous enunciation. Control plates, 22 in number, exposed just before Experiments 19 and 20, were all negative, showing that no serious permanent pollution of the room air had taken place.

In general these results agree closely with those obtained by the German and English observers cited earlier in the paper. The more important data are presented for comparison in Table 8.

TABLE 8.

Comparative Results of Various Observers on Exposure of Plates in Rooms Polluted by Mouth Spray.

Observer	Total Plates Exposed	B. prodigiosus. Mean Colonies per Plate	Percentage of Negative Plates
Laschtschenko	36	17.2	17
Koeniger	321	5.2	39
Gordon	30	0.7	47
Winslow and Robinson	325	2.4	52

Laschtschenko's figures are a little high; but with that exception the results are entirely concordant. It is clear that loud speaking discharges bacteria from the mouth in the form of spray; and that by inoculating the mouth of the speaker with B. prodigiosus and

exposing plates in the room the specific germ may be detected in con siderable numbers.

Suggestive data in regard to the distribution of the mouth spray in space may be obtained by analyzing the results of our experiments according to the distance of the plates from the position of the speaker. The results for each point at which ten or more plates were exposed in different experiments are brought together in Table 9. The heaviest pollution appears to be between 2 meters and 4.5 meters from the speaker. The spray from the mouth probably shoots over areas which are too near; and the particles which are carried beyond 4.5 meters are fewer in number.

TABLE 9.
B. PRODIGIOSUS ON PLATES EXPOSED AT VARIOUS DISTANCES FROM THE SPEAKER.

Distance Meters	Total Plates	Total Colonies	Colonies per Plate
1.0	13	2	.15
1.5	28	62	2.22
2.0	48	199	3.96
2.5	22	126	5.73
3.5	50	208	4.16
4.5	13	40	3.08
5.5	33	61	1.85
7.0	14	32	2.28
7.5	31	42	1.35

Some idea of the general extent of superficial pollution due to mouth spray may be gained from the following considerations. In the room used for all the plate experiments except 7, 9, and 12, 254 plates were exposed and 774 colonies of B. prodigiosus developed, an average of 3.5 colonies per plate. The total area of the room was 480,000 sq.c. and the mean area of a plate 54.5 sq.c. Assuming that all the organisms above a plate settled upon it, the mean number of B. prodigiosus in the whole room in each experiment would be $\frac{480.000 \times 3.5}{54.5}$ or 30,830, and the number discharged on a square meter of surface (by 15 minutes' speaking) would be 646.

QUANTITATIVE METHODS OF ENUMERATING BACTERIA IN THE AIR.

The various processes suggested for enumerating bacteria in the air may be classified broadly under two main heads, filtration methods and sedimentation methods. In one case a measured volume of air is filtered through a powdered solid medium (asbestos, salt, sugar, sodium sulphate, cotton, sand, glass wool) or is bubbled through a

liquid. The solid filtering medium is washed in sterile water and aliquot portions of the water are plated; or portions of the liquid through which the air has been bubbled are plated directly. In all these procedures there is some danger that bacteria may be lost if the medium is not of sufficient fineness, or if the passage of the air is too rapid or if the connections of the apparatus are not all tight. The proper collection of samples with a fine filter on the other hand is time consuming.

The second group of methods involves the exposure of surfaces of nutrient media upon which the bacteria from a known volume of air may settle out and on incubation develop colonies. In its most primitive form ordinary Petri plates are left open in a room for known periods of time. The objection to this is twofold. In the first place the bacteria which fall upon the plates are not related to any determinable quantity of air. In the second place their number will vary greatly with the effect of chance air currents. Hesse long ago founded a more exact method upon this general principle by drawing air slowly through a long cylindrical tube lined with nutrient medium. Recently an improvement on this procedure has been suggested by one of us (Winslow, 1908) involving the use of two liter-and-a-half bottles, having gelatin on the bottom. The bottles are joined by tubing in tandem and a liter of air is drawn in by another water-suction bottle and the bacteria allowed to settle out. The chief objection to this procedure lies in the cumbrousness of the apparatus which requires the carrying about of two large bottles for every sample to be examined. For investigations in the laboratory, however, this procedure has many advantages.

In the present study three methods have been used. Most of the determinations were made by the culture bottle method; and in connection with this procedure two methods of collecting samples have been tried, one the usual method of drawing in air by connecting the second culture bottle with a water-aspirator, the other a vacuum method like that used in collecting air samples for carbon dioxide. In this case the culture bottle is first prepared by covering the bottom with gelatin, plugging with cotton, and sterilizing. At the same time a number of vaselined one-hole rubber stoppers plugged with three-inch lengths of capillary tubing are separately sterilized

in a pipette case. If the bottles were sterilized with the rubber stoppers in them the breakage would be heavy; but after sterilization the stoppers may be substituted for the cotton plugs. Each bottle is then attached by the capillary tube to a suction pump and the air is exhausted for about 10 minutes or until a 90–95 per cent vacuum has been obtained. The capillary tube is now sealed in a flame and the sample of air may be taken at any time by removing the stopper. The danger of leakage from imperfectly fitting stoppers is considerable in this method and many bottles must be discarded but there is a great saving of time in sampling.

Another method used in a few determinations is a simple modification of the plate method designed to secure quantitative results with protection from air currents. An ordinary plate is opened and a covered cylinder a little larger than the plate and about 20 cm. high is placed over it. The apparatus is allowed to stand for half an hour so that the bacteria may settle. Finally in some of the later experiments with mouth bacteria the sand filter method was used, air being drawn by means of a suction bottle through a tandem filter, each member holding a 1.5 cm. layer of sand passing a sieve with 100 meshes to the inch.

QUANTITATIVE STUDY OF AIR POLLUTION BY MOUTH SPRAY USING B. PRODIGIOSUS AS A TEST ORGANSIM.

There are two objections to the method of exposing Petri plates as a measure of true air pollution. In the first place the procedure is necessarily an inaccurate one since the number of bacteria settling is conditioned not only by the number present in the air but also by the amount of motion in the atmosphere. In a strong current of air the plate method must yield results which are relatively too low. On the other hand in quiet air with heavy particles falling through it the results of the plate method must be too high. In the special case with which we are concerned the loud speaking, coughing, or sneezing produces a fine rain which falls more or less rapidly during the period of actual speaking and for a short time afterward. The rate of fall for small falling bodies may be approximately determined from Stoke's law which for spheres of density 1, calling the density of air .00018 and calling g 980, reduces to the form

$$v = 1{,}200{,}000r^2$$

where v is in centimeters per second and r is the radius of the falling particle in centimeters. An ordinary bacterium with a radius of about 0.0001 cm. should therefore fall through quiet air at a rate a little under 0.012 cm. per second, or about 17 inches per hour. If single isolated bacteria were discharged in the mouth spray their fall would be slow and would go on for several hours. As a matter of fact however such is not the case, as is made clear by Koeniger's experiments which have been cited above. He found that 60 per cent of the bacteria in the mouth spray disappeared from the air in 10 minutes, while after 20 minutes less than 10 per cent of the original number remained.

In two of our experiments similar data were obtained by opening all the plates before the speaking but removing some of them after various intervals. The results as shown below indicate no increase in bacteria after the actual period of speaking itself.

TABLE 10.
DURATION OF AIR POLLUTION BY MOUTH SPRAY.
Total B. prodigiosus on plates.

Period of exposure after speaking. Minutes	0	15	30	60
Experiment 12	1	2	..	0
Experiment 23	..	36	34	28

Clearly the mouth spray is a fairly coarse rain which settles out for the most part in 15 or 20 minutes. With such a rain as this the exposure of plates must give an exaggerated idea of the bacterial content of the air. Everything from the air above falls on the plates; but a liter of air should be credited only with the bacteria which it contains at a given moment. Many, even of these, cannot fairly be considered as constituting real aerial pollution if they are in large droplets, for such droplets would be too heavy to be drawn into the mouth with the inspired air.

In order to distinguish between coarse rain falling through the air and finer mist suspended in it, for a period of some minutes at least, we made quantitative determinations by the culture bottle method in Experiments 2, 5, and 6 and in Experiments 15–23, and by the covered plate method in Experiments 7, 8, 22, and 23. In the first three experiments the samples were collected during the speaking by another person. In the last nine the samples were collected just after the

speaking by another person, or by the speaker himself with carefully washed hands. The results of the culture bottle tests are shown in Table 11 below.

TABLE 11.

QUANTITATIVE POLLUTION OF ATMOSPHERE BY MOUTH SPRAY.

Experiment	Duration of Speaking. Minutes	Mean Colonies per Plate Exposed at Same Time	Number of Liter Samples	Number of Samples Showing B. prodigiosus
2	50	..	5	0
5	15	..	3	0
6	35	..	9	0
15	13	0.8	8	0
16	15	2.6	5	0
17	15	6.2	3	0
18	15	13.4	11	1
19	15	7.3	8	0
20	15	5.4	8	0
21	10	10.1	10	2
22	15	4.7	16	0
23	10	2.4	19	0
Total			105	3

Examinations were made by the covered plate method described above in Experiments 7, 8, 22, and 23. Immediately after the close of the speaking the plates were opened and covered with the cylinders which were allowed to stand 30 minutes for the bacteria present to settle. This period is theoretically too short but as a matter of fact the results as shown in Table 12 below were higher than those obtained by the culture bottle method.

TABLE 12.

AIR POLLUTION BY MOUTH SPRAY MEASURED BY COVERED PLATE METHOD.

Experiment	Duration of Reading. Minutes	Mean Colonies per Plate Exposed at Same Time	Number of Samples	Mean Volume of Air Inclosed	B. prodigiosus Found
7	15	0.09	10	840	0
8	15	0.16	11	840	0
22	15	4.7	7	1.370	2
23	10	2.4	7	1,370	2
Totals and averages			35	1,050	4

Altogether taking both quantitative methods into account 140 liters of air were examined and B. prodigiosus was found seven times. The four positive tests with the covered plate were obtained at points about 2 meters in front of the speaker. Two of the positive culture bottles were at the extreme end of the room, nearly 7 meters from the speaker; the other was at a distance of more than 5 meters. These

last results were obtained in the two experiments out of the whole series (see Table 11) which gave the largest number of colonies on the plates.

GORDON'S SPECIFIC TEST FOR AIR POLLUTION.

There is one weak point in all experiments involving an artificial inoculation of the mouth. We have no certainty that the bacterial emulsion introduced in this way behaves as the normal or pathological fluids of the mouth would do. It seems highly probable that a liquid with which the mouth has but just been rinsed would be ejected more freely than the sputum itself. In fact Heymann (1899) and Ziesché (1907) were able to show that in the mouth spray of a consumptive there are several distinct types of droplets which can be distinguished under the microscope and that it is only in droplets derived directly from the bronchi with but little admixture of mouth saliva that the tubercle bacilli are abundantly present. Some more direct test of normal mouth pollution than that furnished by the B. prodigiosus experiments is therefore greatly to be desired.

The real desideratum in the bacterial examination of air is a test which shall quantitatively detect the presence of some normal mouth bacterium which might serve as an index of mouth pollution as the colon bacillus serves in water analysis as an index of intestinal pollution. Such an index has been suggested by Gordon (1904), but its value has not been confirmed by subsequent investigations so far as we are aware. Gordon pointed out that one of the commonest organisms in the saliva is a streptococcus which he called Strept. brevis and which has since been more fully described by Andrewes and Horder (1906) as Strept. salivarius. It is a short chained form which renders broth uniformly turbid, clots milk, reduces neutral red, and ferments saccharose, lactose, and raffinose. Gordon found streptococci capable of fermenting lactose present in from one-millionth to one-hundred-millionth of a cubic centimenter of saliva. Similar streptococci which fail to ferment lactose or ferment neutral red were found to be present in ordinary street air but lactose-fermenting streptococci were absent. Gordon examined 10 samples of 50 liters of street air for streptococci by bubbling it through salt solution which was later mixt with nutrient broth. He found a lactose-fermenting streptococcus only once.

On the other hand, he found that the mouth streptococci could be easily detected in rooms polluted by mouth spray. His method consisted in exposing plates 3.5–5 inches in diameter containing neutral red broth, which was later incubated under anaerobic conditions. In a small room, 4.7 by 4.0 meters in area, 2 out of 6 plates showed mouth streptococci after 15 minutes' loud speaking, 4 out of 6 showed them after half an hour's speaking, and in 5 separate experiments after one hour of speaking 22 out of 30 plates gave positive results. In a larger room, 14.9 by 11.6 meters, 11 out of 34 plates and 28 out of 40 plates were positive after one hour's loud speaking. Quiet speaking and reading yielded negative results. These results coincide closely with those of the German experiments. Like them they measure the superficial discharge of spray but give no clear idea of the quantitative distribution of bacteria in the air above.

QUANTITATIVE STUDY OF AIR POLLUTION BY MOUTH SPRAY USING THE PRESENCE OF MOUTH STREPTOCOCCI AS AN INDEX.

Our final experiments were devoted to the examination of air polluted by mouth spray with a view of determining to what extent the mouth streptococci can be detected in the air itself by ordinary quantatitive methods.

In the first test of this sort the air was examined by the culture bottle method, lactose broth being used in the bottles instead of gelatin. The bottles were placed on a table directly in front of the speaker and from 0.6 to 2.4 meters away. In the first half of the work the speaker's mouth was 0.7 meters above the table and in the second half 1.2 meters above. Five liters of air were first examined for controls. Then the subject orated for 20 minutes, 4 liters of air being examined after each five-minute period. None of the 16 samples showed lactose-fermenting streptococci.

In the second and third experiments the air was examined by the sand filter method. In the second experiment, five different subjects orated loudly for a period of 10 minutes apiece (one taking part twice, so that six samples of air were examined). While the speaking by each subject was going on a sample of 4.5 liters of air was collected by attaching a water aspirator to a tandem sand filter

placed between 0.9 and 1.5 meters away from the speaker and directly in front of him. Each filter in the pair held a layer 1.5 cm. deep of sand which had passed a sieve having 100 meshes to the inch (finer than 0.25 mm.). After collecting the sample the sand was poured into bent dextrose broth tubes and incubated for four days at 37°. Litmus lactose agar plates were made from the broth tubes which showed growth. No lactose-fermenting streptococci were found in any case so that the 27 liters of air examined in this experiment were again all negative.

The third experiment was similar to the second, except that conditions were more severe. Seven tests were made, with two different subjects. The speaking was in English and German and was loud with very vigorous enunciation. In two tests the speaking was interspersed with considerable coughing. Samples of 4.5 liters each were collected during each period of speaking (10 minutes) from a point 35 cm. away from the speaker's mouth and 15 cm. below it. The sand was incubated in dextrose broth for a week, and litmus-lactose plates were inoculated at intervals from the broth tubes. No lactose-fermenting streptococci were found in this examination of 31 liters of air.

Altogether 74 liters of air exposed to pollution by mouth spray were examined by these quantitative methods for mouth streptococci with uniformly negative results. From these experiments it appears, as might be expected, that the pollution of the atmosphere by normal mouth streptococci is considerably less than the contamination with B. prodigiosus after inoculating the mouth with that organism.

Gordon's experiments, made with plates exposed during an hour's reading, of course constituted a very severe test since the plates received all the heavy spray which fell through the air during that period. We made a final experiment along similar lines to his, using litmus lactose agar instead of broth. Eight plates were exposed 45 cm. below and 45 cm. in front of the mouth of the speaker and eight others 15 cm. below and 15 cm. in front. In all these cases the speaking was loud, and vociferous, in English and German, and the period of exposure was five minutes. Finally four plates were exposed 15 cm. in front of and 15 cm. below the mouth of the speaker who orated and coughed for three minutes.

The plates were incubated for four days and subcultures were made from all suspicious colonies. All showed large colony counts (87 to 1,200). Of the first eight plates exposed to spray from speaking 45 cm. away, one only showed a single colony of the acid-forming streptococci. Of the eight plates exposed to spray from speaking 15 cm. in front of and 15 cm. below the mouth, three gave positive results. Two of the plates showed each a single colony, the third was wholly reddened so that the number of acid colonies could not be exactly determined. The streptococcus colonies were not in isolated pure cultures but in mixt colonies each of which was obviously derived from a droplet of saliva containing many forms. Of the 16 plates examined in these two series three were overgrown with an alkalin spreader which might have obscured any streptococci present.

Finally of the four plates exposed to the spray from coughing at a distance of only 15 cm. from the mouth, two were overgrown with alkalin spreaders and so may be excluded from consideration. Of the other two one showed two droplet colonies and the other four. All six on cultivation showed the presence of mouth streptococci.

Altogether it appears from these experiments with the mouth streptococci that normal mouth bacteria are much less easily discharged into the air than are artificial cultures introduced into the mouth. Loud speaking and even coughing did not deposit very large numbers of mouth streptococci even upon plates within 15 cm. of the mouth; and the quantitative examination of 74 liters of air exposed to the spray from loud and vigorous speaking at distances of 35 cm. to 2.4 meters from the mouth failed to show the presence of mouth streptococci in any instance.

SUMMARY AND CONCLUSIONS.

It appears from these experiments, as it appeared in the German investigations which have been reviewed above, that bacteria with which the mouth has been inoculated are discharged in the act of speaking in large numbers, and to considerable distances. Positive results were obtained as far as 7.5 meters from the speaker; and the average number of bacteria discharged over the whole surface of a room (6×8 meters) by 15 minutes' loud speaking was 646 per square meter. These results coincide closely with those obtained by Lascht-

schenko, Koeniger, and Gordon. A still more extensive spread of mouth spray would of course result from coughing and sneezing.

Most of the particles in the mouth spray are rather coarse and settle out rapidly. In fact the spray is like rain falling through the air, rather than mist suspended in it. Koeniger showed that 60 per cent of the bacteria in the mouth spray disappeared from the air in 10 minutes, while after 20 minutes less than 10 per cent of the original number remained. In estimating aerial infection proper, it is the bacteria suspended in the air which are important because these only could be inhaled with the inspired air. Our second object was to attain a quantitative measure of the extent of such aerial infection; and we found that after inoculating the mouth with a rich culture of B. prodigiosus and speaking loudly and with vigorous enunciation for 15 minutes only seven colonies of the specific germ could be obtained from 140 liters of air collected at the close of the speaking from various points in front of the speaker. The true aerial infection is therefore relatively small by comparison with the distribution of the heavier particles of spray.

Experiments with fresh cultures introduced in large amounts upon the surfaces of the mouth give an exaggerated idea of the quantitative importance of the mouth spray. We therefore attempted finally to control the investigation by a study of the distribution of the mouth streptococci which had been suggested by Gordon as offering a promising test of normal mouth pollution. Gordon easily detected these organisms by exposing plates after speaking for various lengths of time. We found them on plates by similar methods; but even at a distance of only 15 cm. from the mouth of a subject who coughed for three minutes the number of droplets containing mouth streptococci was small. Quantitative tests of the air itself from points 35 cm. to 2.4 meters in front of speakers who spoke vigorously in English or German failed to show any mouth streptococci in 74 liters of air examined.

It might be surmised that in pathological conditions bacteria may be more readily dislodged from the mucous membranes than would be the case under normal conditions. Thus the mouth streptococcus may furnish too low an index for measuring the mouth spray, as the B. prodigiosus furnishes too high a one. Experiments carried out with coughing consumptives by Laschtschenko and Heymann, how-

ever, bear out the conclusion that the actual extent of air infection is but small. Out of five air samples of 10 cu.m. each examined by Laschtschenko only two contained tubercle bacilli; out of seven samples of 1 cu.m. each, examined by Heymann, only two gave positive results.

It is not intended to minimize the real danger from mouth spray. Large numbers of bacilli are discharged in coughing by some consumptive patients. Ziesché found in one case 20,000 tubercle bacilli on a glass plate 324 sq.cm. in area exposed for half an hour from 40–80 cm. in front of the mouth of a phthisical patient. The infection by mouth spray of food or of other objects later brought directly or indirectly in contact with the mouth is a real and important danger. Flügge's practical recommendations are fully warranted by all the facts: "During strong paroxysms of coughing, the consumptive should keep at arm's length from his companions and should hold a handkerchief before his mouth. In workrooms, offices, and such places the space between the heads of the workers should be at least 1 meter."

On the other hand all this furnishes no basis for a belief that tuberculosis or any other disease is contracted to an appreciable extent through the inspired air. The mouth spray is a fairly coarse rain which quickly settles downward. Even in artificial experiments with B. prodigiosus the actual extent of air pollution, when quantitatively measured, appears to be small; and when infection by normal mouth streptococci or tubercle bacilli is studied it is much smaller still.

These conclusions are in harmony with the conviction now generally gaining ground that aerial infection of any sort is a minor factor in the spread of zymotic disease; and in regard to tuberculosis they accord well with the opinion that ingestion rather than inhalation is the principal channel of infection even in the case of bacilli originally discharged through the air in the form of mouth spray.

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THE BACTERIAL FLORA OF MILK HELD AT LOW TEMPERATURES.*

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The value of milk and cream for human food, either alone or as constituents of more elaborate dishes, has led to efforts for their preservation for considerable periods of time. Such preservation is necessary in order to furnish a supply for places where the fresh product cannot be obtained, as on ocean steamships, or for a time of abnormal demand, such as that occasioned by the Fourth of July. The bacterial changes undergone by milk during its preservation at low temperatures have been made the subject of considerable study by Conn and Esten,[1] and by Pennington,[2] these investigators being preceded by Havemann,[3] Schmidt-Nielson,[4] Schmelk,[5] Conradi and Vogt,[6] Fischer,[7] and Muller,[8] who showed that certain bacteria can grow at a temperature as low as 0° C. The investigations of these earlier scientists consisted mainly in keeping various inoculated menstra at about 0° C. and making quantitative bacterial determinations at stated times. Their conclusions were that, altho growth does take place, it is comparatively slow.

Conn and Esten showed that at 1° C. there is a period of from six to eight days during which there is no increase in the total number of bacteria in milk, and that when growth does start at this temperature the lactic bacteria fail to predominate over the other species, so that all types develop. They concluded that milk is not necessarily wholesome because it is sweet and that the species developing at this low temperature are more apt to be injurious than species developing at 20° C. Pennington concluded that at the low temperature (29–32° F.) employed in her experiments there were constantly present bacteria which formed acid and bacteria which acted on protein, the former

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1 *Report Storrs Agri. Exper. Sta.*, 1904, p. 27.

2 *Jour. Biol. Chem.*, 1908, 4, p. 353.

3 *Ueber das Wachstum von Mikroorganismen bei Eiskrank Temperature*, Inaug. Dis., Rostok, 1894.

4 *Centralbl. f. Bact.*, 1902, 9, Abt. II, p. 145.

5 *Ibid.*, 4, Abt. I, p. 545.

6 *Ztschr. f. Hyg.*, 1899, 331, p. 287.

7 *Deut. med. Wchnschr.*, 1893, 19, p. 598.

8 *Arch. f. Hyg.*, 1903, 47, p. 127.

being in relatively smaller numbers and the latter in relatively higher numbers than in milk kept at a higher temperature. Chemical study showed that the casein was rapidly digested until more than 50 per cent of it was changed to soluble compounds apparently caseoses, amino acids, and probably peptones.

The experiments herein detailed were undertaken with the intention of studying the activity of the bacterial flora of milk and water kept at both $-9°$ and $0°$ C. Two milks were used, one, referred to as Barn milk, was supplied by the university herd; while the other, designated Dairy milk, was obtained at the university creamery, being a mixt milk supplied by the farmers about the city. The Barn milk was considered to be the best milk obtainable; it is produced under very good conditions and the average bacterial count is about 3,000 per c.c., with a low percentage of acid formers. The Dairy milk was taken because it was considered to be a fair dairy sample produced under average conditions; the bacterial count is quite variable with a rather large percentage of acid formers. The water used was that supplied to the various laboratories at the University of Wisconsin. The source is a lake of about 30 square miles in area.

At the beginning of the experiment the only medium used was a 2 per cent lactose agar having an acidity of 0.5 per cent, the plates being incubated at $37°$ C. Later the idea of using a medium somewhat more differentiating in character presented itself and a 2 per cent lactose gelatin with an acidity of 1 per cent was employed, the gelatin plates being incubated at $12°-15°$ C. These two media were then employed to the close of the experiment.

The containers used were sterile 8 oz. bottles. These were filled to within 25 c.c. of their capacity, in the case of the Barn milk immediately after the milk was drawn, while in the case of the Dairy milk at once upon arrival of the milk at the creamery. The bottles were then stoppered with sterile corks and sealed with paraffin. The specimens so prepared were at once packed in ice, repacked in ice and shavings in about two hours, and shipped to Waterloo 22 miles distant, where they were immediately freighted to the cold storage plant, unpacked, and stored in their respective cold rooms.

In order to see what effect the transportation would have on the

bacterial content of the specimens, one bottle of each kind of milk was plated before repacking in ice and shavings, and one bottle of each was brought back from Waterloo the same day and plated. These results are given in the following table:

TABLE 1.
Effect of Shipment on Bacterial Content of Milk.

	No. per c.c. before Repacking	No. per c.c. after Transportation
Barn milk	3,500	5,300
Dairy milk	130,000	175,000
Water	68	29

This indicates that the transportation has only an insignificant effect on the bacterial content of the milks in question.

SPECIMENS KEPT AT $-9°$ C.

The specimens were placed in the cold rooms August 6, 1908, and from that time on bottles were brought to the laboratory and plated at various times with the following results:

TABLE 2.
Bacterial Content at Various Intervals of Specimens Held at $-9°$ C. Plated on Lactose Agar and Incubated at $37°$ C.

Date	Age of Milk in Days	Water	Dairy Milk	Barn Milk
8-6-'08	0	68	130,000	3,500
8-12-'08	6	15	108,500	6,400
8-21-'08	15	16	95,500	5,850
8-26-'08	20	9	92,000	5,250
9-11-'08	36	9	111,500	3,050
9-11-'08	36	17	100,500	1,200
10-19-'08	74	26	127,000	5,000
11-20-'08	106	16	63,750	1,950
1-13-'09	160	5	51,000	....

TABLE 3.
Bacterial Content at Various Intervals of Specimens Held at $-9°$ C. Plated on Plain Gelatin and Incubated at $12°$–$15°$ C.

Date	Age of Milk in Days	Water	Dairy Milk	Barn Milk
8-21-'08	15	14	8,600,000	3,400
8-26-'08	20	28	3,200,000	6,250
9-11-'08	36	30	600,000	2,650
9-11-'08	36	26	650,000	3,600
10-19-'08	74	16	145,000	5,750
11-20-'08	106	10	65,000	3,200
1-13-'09	160	5	57,000	....
2-25-'09	203	16	25,300	1,650

Milks.—All the bottles kept at $-9°$ C. remained in a frozen condition until brought to the laboratory and here they were thawed

just previous to plating. After six days the casein and fat were thrown into clumps that could not be again incorporated into the milk serum; this condition caused considerable difficulty in sampling and introduced an exceptionally large experimental error. At this time the odor and taste in both specimens were those of good milk and these conditions persisted for 36 days, when there was a decided watery taste in each case, while on standing over night, after being melted, the casein would be at the bottom of the bottle with a watery layer above. After 74 days the Dairy milk was slightly off in flavor altho the Barn milk was not, but after 106 days, both specimens were off in odor and taste; the odor was not suggestive of putrefaction, but was decidedly undesirable. The number of organisms developing at 37° C. on agar remains fairly constant, the variations evidenced being insufficient to allow of any definite conclusions. After 36 days storage, duplicate bottles were brought to the laboratory and plated and the results given in the table show that the differences between bottles of the same milk are negligible and quite within the limit of experimental error.

The lack of gelatin counts early in the experiment leaves a considerable gap in the results, but the data show that while the number of organisms in Barn milk developing on this medium remains fairly constant, the number in Dairy milk diminishes considerably.

The acidity of each specimen of milk was determined as soon after reaching the laboratory as possible and the data are presented in the following table:

TABLE 4.
Acidity of Milks Held at −9° C.

Date	Age of Milk in Days	Dairy	Barn
8– 6–'08	0	.16	.18
8–12–'08	6	.15	.18
8–21–'08	15	.16	.18
8–26–'08	20	.15	.18
9–11–'08	36	.14	.17
9–11–'08	36	.14	.16
10–20–'08	74	.13	.15
11–20–'08	106	.11	.09
1–13–'09	160	.11	.10
2–25–'09	203	.10	.10

From these results we see that there is a decrease in acidity and this was, of course, due either to a purely chemical, or to a biochemical change. The latter possibility presupposes a change in the bacterial

flora of the milk; such a change cannot be quantitative, as has been shown by our determinations, altho there is, of course, the possibility that the determining organisms in this reaction-change do not develop on the media used. It seems more probable that the change is purely chemical and altho as yet we have no data to prove this hypothesis, experiments are at present under way which will furnish evidence along this line.

The milks examined after 36 and 203 days storage were left at room temperature for two and four days respectively and then titrated again, with the idea of finding out whether or not there would be an increase in acidity.

TABLE 5.
Increase in Acidity Due to Influence of Higher Temperature.

Sample	9–11–'08	9–13–'08	2–25–'09	3–3–'09
Barn	.17	.43	.10	1.71
Dairy	.14	.62	.10	1.11

This table shows that when the milks are exposed to room temperature the acid-forming bacteria again gain the supremacy over the other forms and the acidity of the specimen increases.

On the milks brought to the laboratory after 203 days storage, total nitrogen and water soluble nitrogen determinations were made. Because of the physical condition of the milks it was a difficult matter to obtain a sample and the method finally decided upon was to pipette off 5 c.c., using a pipette with a 3 mm. opening, for the total nitrogen determination. In the case of the water soluble nitrogen a 10 c.c. sample was placed in a beaker, diluted to 50 c.c. with water, a small amount of acetic acid added, and the resulting material heated on a water bath for some time, after which the soluble nitrogen compounds were allowed to filter into a Kjeldahl flask. The results given in the following table are the averages of duplicates which agreed very well:

TABLE 6.
Nitrogen Distribution in Milks Held at −9° C.

	Percentage Total Nitrogen	Percentage Insoluble Nitrogen	Percentage Soluble Nitrogen	Percentage of Total Nitrogen Present as Soluble Nitrogen
Barn −9°	.5375	.4409	.0966	17.97
Dairy −9°	.5161	.4006	.1155	22.38

In comparison with fresh milk, which shows about 10 per cent

of its nitrogen in a soluble form, we see here a percentage almost double that in fresh milk. This is probably due to enzyme action as this interpetation agrees very well with the data obtained at the Wisconsin Experiment Station during the study of galactase.[1]

Water.—The data obtained in the case of the water held at $-9°$ C. shows that at this temperature there is no increase in the number of organisms developing on either lactose agar or gelatin.

SPECIMENS KEPT AT 0° C.

These bottles were also refrigerated August 6, 1908, and were plated on the same days as the specimens kept at $-9°$. The results are given in the following tables:

TABLE 7.

BACTERIAL CONTENT AT VARIOUS INTERVALS OF SPECIMENS HELD AT 0° C. PLATED ON LACTOSE AGAR AND INCUBATED AT 37°.

	Age of Milk in Days	Water	Dairy Milk	Barn Milk
8-6-'08	0	68	130,000	3,500
8-12-'08	6	12	72,500	4,050
8-21-'08	15	22	633,500	52,900
8-26-'08	20	21	3,230,000	1,240,000
9-11-'08	36	14,160	34,950,000	4,800,000
10-19-'08	74	33,300	91,500,000	36,500,000
11-20-'08	106	8,000	39,750,000	192,500,000
1-13-'09	160	2,300	32,650,000	361,000,000

TABLE 8.

BACTERIAL CONTENT AT VARIOUS INTERVALS OF SPECIMENS HELD AT 0° C. PLATED ON PLAIN GELATIN AND INCUBATED AT 12°–15° C.

	Age of Milk in Days	Water	Dairy Milk	Barn Milk
8-21-'08	15		10,950,000	252,000
8-26-'08	20		53,467,000	2,765,000
9-11-'08	36		160,000,000	39,000,000
10-19-'08	74	225,000	135,000,000	111,500,000
11-20-'08	106	165,500	88,000,000	229,500,000
1-13-'09	160		94,500,000	191,500,000
2-25-'09	203		65,500,000	183,000,000

Milks.—These samples never showed any ice formation, altho a dish of water standing beside the bottles developed a scum of ice. The specimens brought back after 36 days were the first to show an off flavor; this was but slight in the Barn milk, but in the Dairy milk it was very decided, the odor in each case, however, being about normal. On this date the bottle of Dairy milk showed a clumping of the casein, altho this was not as evident as in the milks at the lower temperature.

[1] *Wisconsin Experiment Station, Twentieth Rept.*, p. 201.

The bottles brought back after 106 days both gave an appearance of digestion, and after shaking and drawing the cork there was a vigorous evolution of bad smelling gases.

The Dairy milk showed a steady increase in organisms developing at 37° after a period of apparent quiescence. Later the decrease in bacteria was marked. The Barn milk, after the quiescent period, showed a steady and marked increase in bacteria. On gelatin, so far as the incomplete data show, it appears that much the same conditions obtained with each kind of milk—at first a great increase in numbers of bacteria, followed by a decrease.

The percentage of acid noted in the various samples of milk at the periods of observation are given in Table 9.

TABLE 9.

Percentage of Acid of Milks Held at 0° C.

Date	Age of Milk in Days	Dairy	Barn
8-6 -'08	0	.16	.18
8-12-'08	6	.15	.18
8-21-'08	15	.17	.19
8-26-'08	20	.17	.19
9-11-'08	36	.21	.21
9-11-'08	36	.21	.20
10-20-'08	74	.54	.32
11-20-'08	106	.65	.58
1-13-'09	160	.92	.91
2-25-'09	203	.68	.77

This shows a considerable increase in acidity for each specimen, but no plates with a marked lactic odor or with any considerable number of lactic appearing colonies were encountered. From this it is evident that the lactic acid organisms did not overcome the other organisms and that the acidity to some extent at least was produced by organisms other than lactic.

The distribution of the nitrogen was determined in the specimens brought to the laboratory after 203 days storage with the following results:

TABLE 10.

Distribution of Nitrogen in the Specimens Held at 0° C.

	Percentage Total Nitrogen	Percentage Insoluble Nitrogen	Percentage Soluble Nitrogen	Percentage of Total Nitrogen Present as Soluble Nitrogen
Barn 0°	.360	.098	.262	72.78
Dairy 0°	.3966	.111	.2856	72.01

The digestion of nitrogenous bodies herein shown can hardly be attributed to the enzymes inherent in the milk itself and must therefore necessarily be attributed to bacterial action. The most peculiar thing about the above results is the marked loss in total nitrogen which can best be explained by assuming a liberation of free nitrogen. This is quite probable because it was observed that on opening the flasks held at this temperature there was a vigorous evolution of gas which was not at all suggestive of ammonia.

Water.—The specimen of water kept at this temperature showed a period of quiescence when first refrigerated similar to that exhibited by the milks, but this persisted for a longer period in the case of the water. The rise was so rapid in the organisms developing on gelatin that no count was obtained for a considerable period because of the low dilutions. The small amount of data at hand evidently show a decrease finally and this decrease is also marked in the organisms developing at 37°.

CONCLUSIONS.

In milks held at $-9°$ C. there was a clumping of the casein and fat, no increase in bacteria developing on agar and gelatin, and an increase in the amount of soluble nitrogen and a decrease in the acidity.

In milks held at 0° C. there was a marked increase in the bacterial content resulting in an increase in acidity, an increase in the percentage of soluble nitrogen so that it eventually amounted to over 70 per cent of the total nitrogen, and a decrease in the total nitrogen content probably due to a liberation of free nitrogen.

This work has especial importance in connection with the handling of one dairy product, viz., cream. The amount of cream received by the city milk dealer does not vary widely from day to day, while the demand for it varies, being influenced by temperature conditions, occurrence of holidays, etc. The storage of cream for considerable periods is therefore practiced. As is shown by the data presented, the temperature of storage, 33 to 40° F., is such as to exclude the growth of the ordinary lactic bacteria, but not the growth of the putrefactive organisms.

The cream may be normal in appearance and taste but it may contain the by-products of the putrefactive bacteria in such amounts

as to render it harmful. Poisoning by ice-cream is quite frequent, by milk very rare indeed. The question may be raised: Could not many of the cases of ice-cream poisoning be traced to the use of cream in which, during prolonged storage, toxic products have been formed?

In pasteurized milk the lactic bacteria are usually absent, a free field is left for the putrefactive spore-forming bacteria, whose action on the milk may not be apparent to the consumer, and yet the milk may be distinctly harmful.

In practical effects cold storage is identical with pasteurization since it removes from the sphere of action that class of bacteria that prevent the growth of harmful kinds of organisms, while it allows the harmful bacteria to develop.

ON THE PRODUCTION OF SANITARY MILK.*

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The experiments reported in this paper were undertaken with a view to arriving at definite conclusions in reference to some stages in the production of sanitary milk, or so-called "certified milk." The results shall be discussed under four heads, with an added fifth part for a short criticism of dairy score cards. The four experimental parts are:

1. A study of the bacterial content of separator milk and cream.

2. Comparative tests of the value of narrow top pails with and without strainers.

3. A study of the action of absorbent cotton strainers on the bacterial content of milk.

4. An attempt at classification of body cells in the slime of the separator.

The experiments were carried on during the month of September, 1909, at a model dairy in Wisconsin.[1] All conditions and precautions for the production of sanitary milk are carried out here to the smallest detail. All utensils are thoroughly sterilized, the separator in the bottling-room is sterilized with live steam before use, the bottling-room is finished with glazed tile in such fashion as to offer smooth surfaces for the play of steam, and the cows are in perfect health. We were thus enabled to obtain samples and carry on the work with the assurance that the possibilities for contamination were minimized and that the milk was the natural product of healthy cows.

Methods.—The samples were taken with sterile pipettes from the milk after thorough mixing, excepting the samples from the separator milk and cream, which were taken while the milk and cream were flowing from the separator. The contents of the pipettes were discharged into tubes containing 9 c.c. sterile water. One c.c. of this dilution was mixt in a petri dish with the contents of a tube of liquefied beef-extract agar, containing 1 per cent glucose and having a reaction of 1 per cent acid. The

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[1] We are greatly indebted to the owners and the manager, Mr. A. J. Obenberger, of the Edgewood Farms at Pewaukee, Wis., for putting material, apparatus, and space at our disposal, and for the courtesy and hospitality shown us during the progress of the work.

plates were kept in a dark box at room temperature. Frequent observations of the temperature showed that it varied but little, averaging about 18° C. The colonies were counted after three days with a lens magnifying about five times. The samples for the experiments under 2 and 3 were taken from the morning's milking at 5 A. M. and all plating was finished at about 7 A. M. or a little later. The samples for experiments 1 and 4 were taken from the separator milk and cream at about 4 P. M. and also plated immediately. It is the usual experience in sanitary dairies that the morning milk contains somewhat higher numbers of bacteria than the afternoon milk, this probably being due to the fact that the general condition of the stables, after the cows have passed the night indoors, is not as good as it is after the stables and cows have been thoroughly cleaned.

A STUDY OF THE BACTERIAL CONTENT OF SEPARATOR MILK AND CREAM.

This subject has received the attention of some investigators, but the opinions of these are at variance with each other. Wyss[1] thought that a large number of bacteria were thrown out into the separator slime. He also stated that the milk is purified by the process of separation. Scheurlen[2] claimed that the centrifugal cream as well as gravity cream contains more bacteria than the milk before centrifugation. He worked with 20 per cent cream. Niederstadt[3] came to the conclusion that the cream contains 75 per cent of all bacteria originally found in the milk and the separator milk only 25 per cent. Rolet[4] found, after centrifuging five samples of milk, that on the average the cream contained slightly more bacteria than the milk. Russell[5] states that gravity cream is usually richer in bacteria than separator cream, but that this is mainly due to the age of gravity cream. Both kinds of cream according to this author contain more bacteria than the milk from which they are obtained. He also states[6] that separator cream usually contains larger numbers of bacteria than the separator milk. Russell and Hastings[7] make substantially the same statements. Anderson[8] also found that both gravity and separator cream contain more bacteria than the milk. Wilkens[9] on the other hand stated that centrifugalized milk contains

[1] *Centralbl. f. Bakt.*, 1889, 6, p. 587.

[2] *Arbeit. a. d. kais. Ges.*, 1891, 7, p. 269.

[3] *Koch's Jahresber.*, 1893, 4, p. 205.

[4] *Ibid.*, 1901, 12, p. 340.

[5] *Outlines of Dairy Bacteriology*, 1894, pp. 126, 127.

[6] *Ibid.*, p. 125.

[7] *Experimental Dairy Bacteriology*, 1909, p. 93.

[8] *Jour. Infect. Dis.*, 1909, 6, p. 392.

[9] *Centralbl. f. Bakt.*, Ref., 1894, 16, p. 969.

less bacteria than the original milk, but agrees with the other authors cited, that the majority of bacteria go into the cream. He ascribed the reduction of bacteria to the violent rotation of the centrifuge, in consequence of which, he thought, many bacteria are killed.

Wholly different results were obtained by Backhaus and Cronheim.[1] These authors found that the total number was actually decreased by centrifugation, but that the majority of bacteria go into the skim milk. They also showed that 95.6 per cent of the dirt in the milk was thrown out by the separator. Swithinbank and Newman[2] admit that gravity cream contains large numbers of bacteria, but state that separator cream contains few. "Roughly 60 per cent of the organisms will be found in the bowl sediment (separator slime), 25 per cent in the separated milk, and 15 per cent in the separated cream." They also quote Eckles and Barnes as having estimated the numbers of bacteria as follows: 47 per cent in the slime, 29 per cent in the separated milk, and 24 per cent in the separated cream. Careful experiments were reported by Severin.[3] He found that the number of bacteria in the milk after separation and subsequent mixing was always larger than in the original milk, in spite of the fact that the dirt was removed. He made experiments to show that contamination from the air or some other source was not responsible for the increase. As an explanation he offered the theory that since bacteria in milk occur in clumps, or chains, or in imperfectly separated pairs, these aggregates are broken up by the force of the centrifuge and a larger number of colonies results. Russell[4] explained the occurrence of larger numbers of bacteria in centrifugal cream by the supposition that the bacteria are carried mechanically by the cream to the center of the separator.

We made a series of experiments to test the number of colonies obtainable on glucose agar from the milk as it enters the separator and then from the cream and the skim milk after leaving the separator. The contents of two large cans of milk were separated daily, the process occupying about 20 minutes. We took samples from each part at intervals of five minutes. This work was continued for

[1] *Koch's Jahresber.*, 1897, 8, p. 151.

[2] *Bacteriology of Milk*, New York, 1903, p. 144.

[3] *Centralbl. f. Bakt.*, Abt. II, 1905, 14, p. 605.

[4] *Outlines of Dairy Bacteriology*, 1894, p. 134.

16 successive days. The results are given in Table 1 in detail and the averages in Table 2. The original milk as appears from Table 2 averaged 738 bacteria per c.c. for the total of 48 samples examined. The separator milk for the same number of tests and taken as the milk flowed from the separator contained 2,130 bacteria per c.c.,

TABLE 1.

The Number of Colonies in Milk before and after Passing through the Separator.

Day	Milk before Separating			Separator Milk			Separator Cream			Calculated Number of Bacteria after Separating and Mixing		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
1	4,110	820	1,050	12,930	25,530	5,910	190	70	170	12,060	23,800	5,520
2	8,800	250	760	14,100	1,980	1,040	580	40	280	13,050	1,830	780
3	1,460	1,770	2,440	1,280	4,420	830	170	310	680	1,200	4,110	820
4	380	490	410	510	610	460	190	310	260	490	590	440
5	370	810	140	11,000	850	970	80	70	30	10,140	590	900
6	330	610	230	640	280	2,090	90	80	90	600	270	1,950
7	220	80	90	870	80	130	230	170	120	820	90	130
8	910	490	960	2,110	360	130	120	120	130	1,990	350	130
9	990	380	730	2,190	330	270	80	110	80	2,040	310	260
10	170	470	310	2,240	1,790	190	150	150	40	2,080	1,670	180
11	1,750	950	790	1,510	1,020	1,770	180	170	120	1,420	960	1,660
12	60	110	170	430	160	230	80	160	30	410	160	210
13	50	30	140	80	170	260	30	80	100	80	160	250
14	130	30	20	50	130	20	20	10	60	50	120	20
15	50	30	20	140	30	30	20	30	30	130	30	30
16	70	60	50	50	30	30	10	20	20	50	30	30
Total	19,750	7,380	8,310	50,130	37,770	14,360	2,220	1,900	2,240	46,610	35,270	13,510
Average	1,234	461	519	3 133	2,361	898	139	119	140	2,913	2,204	844

TABLE 2.

Totals and Averages of 48 Samples of Milk before and after Passing through the Separator.

	Milk before Separating	Separator Milk	Separator Cream	Mixt Milk
Sample 1	19,750	50,130	2,220	46,610
Sample 2	7,380	37,770	1,900	35,270
Sample 3	8,310	14,360	2,240	13,510
Total	35,440	102,260	6,360	95,390
Average	738	2,130	132	1,987

and the separator cream, which tested about 40 per cent fat, had the small number of 132 bacteria per c.c. Taking the sum of the numbers found in the separator milk and separator cream as a basis of calculations we found 17 per cent of the bacteria in the cream. As it is difficult to estimate the number in the separator slime, such an estimation would not add to the accuracy of this figure, but would reduce the percentage of bacteria in cream materially. In

the last column of the tables the numbers of bacteria are calculated according to the numbers found in the cream and skim milk, as they would appear if the cream and skim milk were mixt again after separation.

Since the conditions under which the separation took place were above reproach as far as cleanliness and handling were concerned, we are forced to the same conclusion as Severin, namely, that the larger number of colonies appearing after separation are due to bacteria originally contained in the milk. Our results seem, therefore, to bear out the theory that clumps and aggregates of bacteria which are in the milk are broken up by the violence of the process of centrifugalizing, thus increasing not the actual number of bacteria, but the number of colonies appearing on the plates. The exceedingly small number of bacteria in the centrifugal cream we can account for only on the assumption that the majority of bacteria are thrown into the milk and slime. It is a well-known fact that the aggregates of fat globules are largely broken up by the force of the separator, in consequence of which fact milk obtained by the mixing of centrifugal milk and cream shows a poor cream line, the cream rising more slowly than in milk which has not been separated. On account of this breaking up of the clumps of fat globules we believe that on the whole bacteria are not carried to the center of the centrifuge but go to the outside into the skim milk and the slime.

In gravity cream it is generally accepted that bacteria are carried to the top with the cream. In this case, the fat globules not having been broken up, the theory is more acceptable. Still, the larger number of bacteria in gravity cream can be plausibly explained on Russell's assumption that gravity cream is necessarily older than separator cream.

As stated before, the cream contained about 40 per cent fat. It is the custom of many dairies, in order to separate a rich cream, to test the exact percentage of fat, and then dilute it with skim milk until the desired amount of fat has been established. In certified milk, which is largely used for infant feeding, the usual amount of fat is 16 and 32 per cent. To judge from our experiments this mixing of separator milk and cream results in a cream having a larger number of bacteria than the original milk, since we found that the separa-

tor milk contained many more bacteria per c.c. than the whole milk. The question then confronts us, whether the number of bacteria would not be smaller if, instead of separating a cream with a high percentage of fat, the cream was separated directly with the desired amount of fat, so as to obviate the necessity of diluting it with separator milk. Experiments to determine this point are now in progress. A cream with smaller numbers of bacteria would undoubtedly be obtained, if after separation whole milk was used for dilution instead of skim milk. Obviously this cannot be done at the time the milk is modified, since whole milk contains about as much fat as it is practicable to have in modified milk and cannot serve as a diluent.

COMPARATIVE TESTS OF THE VALUE OF NARROW TOP PAILS WITH OR WITHOUT STRAINERS.

The milking pail which was used for these experiments has an opening of about six inches diameter. Into this opening is fitted a cylindrical piece with a brass sieve at the lower end. The sieve has 100 meshes to the inch. By lifting out this cylinder the milk can be poured into a larger can without the necessity of a spout on the side such as the Gurler pail has. This style of pail is easily cleaned and sterilized.

Theoretically it might be argued that the presence of a strainer or sieve might increase the number of bacteria, since some dirt, which would gather on the strainer, would be broken up by the force of the streams from the teats. Stocking[1] came to the conclusion that in most cases the strainer affords a distinct advantage and that the relative advantage gained depends upon the sanitary condition of the stable. It seems reasonable, therefore, to conclude, that the more perfect the precautions applied, the less will be the difference in bacterial counts obtained from milking into a pail with or without a strainer.

We carried out a series of 108 tests, each with and without the strainer, under the following conditions: Twelve cows were selected and a sample taken in the manner described above from the total yield of one milking of each cow. The strainer was used on alternate days so that the result represents the number of colonies per c.c.

[1] *Storrs Agric. Exp. Sta. Bull.*, 48, 1907.

obtained from one morning's milking with the strainer and the next day without the strainer. We realize that the bacterial content of milk from the same cow may vary from day to day, but we consider the results as representing the actual conditions fairly because of the large number of tests. Table 3 shows the figures in detail and Table 4 gives a summary and the averages. The experiments

TABLE 3.

COMPARATIVE NUMBERS OF COLONIES PER C.C. OF MILK RECEIVED IN SMALL TOP PAILS WITH AND WITHOUT STRAINERS.

No. of Sample	No. of Cow 2		No. of Cow 3		No. of Cow 5		No. of Cow 9		No. of Cow 13		No. of Cow 15	
	Str.	Open	Str.	Open	Str.	Open	Str.	Open	Str.	Open	Str.	Open
1	390	190	240	820	280	770	580	670	310	170	560	140
2	2,320	110	80	1,300	240	6,310	470	2,160	240	380	110	2,860
3	6,540	290	2,960	20	170	30	940	70	40	40	9,600	540
4	10	20	40	80	40	40	120	960	60	70	80	90
5	20	150	80	20	130	90	210	220	370	10	160	510
6	190	2,100	90	1,210	1,690	360	2,070	1,140	470	90	90	310
7	190	70	170	40	150	40	140	70	150	570	180	70
8	20	10	10	110	30	10	70	20	210	90	10	10
9	80	20	40	20	30	90	160	40	70	50	40	350
Total	9,760	2,960	3,710	3,620	2,760	7,740	4,760	5,350	1,920	1,470	10,830	4,880
Average	1,084	329	412	402	307	860	529	594	213	163	1,203	542

No. of Sample	No. of Cow 18		No. of Cow 21		No. of Cow 22		No. of Cow 27		No. of Cow 29		No. of Cow 38	
	Str.	Open	Str.	Open	Str.	Open	Str.	Open	Str.	Open	Str.	Open
1	230	420	310	390	1,770	660	690	410	2,650	7,500	1,420	310
2	990	510	650	210	1,940	2,400	810	2,100	5,730	1,800	1,350	3,200
3	20	20	260	520	920	590	50	130	690	510	130	110
4	140	120	120	1,990	330	510	120	40	1,420	2,050	70	160
5	390	490	410	70	440	1,300	170	190	2,390	670	570	520
6	90	2,110	130	210	1,440	1,290	480	3,010	570	8,690	1,190	470
7	110	80	140	110	490	230	70	20	170	20	170	60
8	60	70	10	20	40	30	120	40	10	40	40	30
9	30	40	440	30	110	60	20	30	30	160	20	80
Total	2,060	3,860	2,470	3,550	7,480	7,070	2,530	5,970	13,660	21,440	4,960	4,940
Average	229	429	274	394	831	786	281	663	1,518	2,382	562	549

were begun early in September and continued throughout the month. At first the weather was rather warm but soon turned cool, and in accordance with this change we find a more or less gradual falling off in the number of colonies. The variation in numbers of colonies in milk from the same cow from day to day is considerable; still there seems to be a certain amount of consistency, the milk from some cows having a lower bacterial content than that from others.

From Table 4 we see that the average of colonies obtained from the milk in open pails is 674 per c.c., while the average of the milk obtained through a sieve is 620. There is, therefore, a slight advantage in favor of the use of the sieve, altho the difference is small and possibly ranges within experimental error. We have come to the conclusion for reasons which will appear later that it is advisable to retain the strainer in the milking-pail, especially in dairies where conditions are not ideal. If the animals are not kept as scrupulously clean as they are kept in the dairy where these experiments were carried on, it is hardly possible to avoid having small particles of dirt,

TABLE 4.

Totals and Averages of Bacteria in Milk from Pails with and without Strainers.

	Pails with Strainer	Pails without Strainer
	9,760	2,960
	3,710	3,620
	2,760	7,740
	4,760	5,350
	1,920	1,470
	10,830	4,880
	2,060	3,860
	2,470	3,550
	7,480	7,070
	2,530	5,970
	13,660	21,440
	4,960	4,940
Total	66,900	72,850
Average	5,575	6,071
Average per cow	620	674

hairs, etc., fall into the milk and the strainer will keep these out. If there is truth in the supposition that particles of dirt may be broken up by the force of the stream from the teats during milking, this may perhaps be avoided by having a conical sieve instead of a level one, so that the dirt particles are washed down to the edge of the sieve where the force of the milk streams has been largely broken.

A STUDY OF THE ACTION OF ABSORBENT COTTON STRAINER ON THE BACTERIAL CONTENT OF MILK.

In many dairies the milk from the individual cows is collected in large cans and then carried to a room for straining through cotton before reaching the bottling-room. At the Edgewood Farms a reservoir of the following construction is used for this purpose:

The strainer frame, set on a large cylindrical tank, is 14 inches high and 13 inches in diameter. In the bottom of this strainer frame there is a brass strainer of 100 meshes

to the inch. This prevents loose parts of the cotton from being carried into the strained milk. Above this brass strainer there are three separate wire nets of two wires to the inch and upon each of these nets rests a layer of sterile absorbent cotton. Above the cotton there is a steel plate with 97 holes, each opening being $\frac{3}{16}$ of an inch. This steel plate is designed to break the force of the stream of milk before reaching the cotton. The strained milk reaches the cooler from the reservoir by means of a pipe, which is taken apart after each milking for sterilization.

We made tests to find whether the number of bacteria was reduced or increased in milk by straining in the manner described above. Samples were taken at intervals of 25 minutes, first from the milk in the can, then from the top of the strainer, and finally from the milk when flowing on the cooling apparatus. Thus we obtained five samples at each stage of the process, altogether 15 samples daily, and this scheme was continued for 16 days. The technic of sampling and plating was the same as in the former series of experiments. The results are tabulated in Tables 5 and 6.

The milk before straining contained an average of 2,060 bacteria per c.c. On top of the strainer the number had increased to 3,912, and after passing through the cotton the number was 2,790. There is then first a decided increase on the strainer. Possibly this increase is due to the violent breaking up of clumps of bacteria as a result of pouring a large amount of milk on top of the strainer. This theory receives support from the fact that as the time advanced the numbers increased materially, so that the sample taken 25 minutes after the first one contained more bacteria than the first sample, and 25 minutes later the number was still larger, until after the last sample was taken, that is to say, after milk has been poured on the strainer for about two hours, the number reached the highest point.

The strained milk contained less bacteria than the milk on top of the strainer, but more than the milk in the can before straining. It is, then quite evident that the straining through cotton removes some of the bacteria, but this reduction does not compensate for the numbers resulting from the breaking up of clumps, etc., on top of the strainer. The result would, therefore, be more favorable if this straining process was omitted.

Since it is practically impossible to prevent hairs, dirt, and other foreign material, covered more or less with bacteria, from occasionally gaining access to milk, it seems advisable that at some period or

TABLE 5.

NUMBER OF COLONIES IN 1 C.C. OF MILK BEFORE AND AFTER STRAINING THROUGH COTTON.

DAY	MILK IN CAN BEFORE STRAINING					MILK ON TOP OF STRAINER					MILK AFTER STRAINING				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
1	2,790	510	2,830	660	1,840	1,389	460	1,210	1,820	1,170	2,650	1,050	1,770	610	1,790
2	630	340	2,510	9,100	610	570	620	2,400	31,000	27,000	150	6,100	1,900	14,800	2,230
3	2,050	1,750	23,220	3,840	730	1,550	1,420	51,400	1,340	21,100	1,070	780	3,010	15,900	29,900
4	12,100	4,100	1,220	30,720	1,320	5,500	2,520	2,150	1,990	970	3,160	4,610	2,500	6,250	910
5	2,280	240	2,660	290	15,000	2,120	21,000	4,900	3,880	3,220	1,670	450	1,380	240	270
6	350	80	810	140	420	260	60	930	70	260	280	130	660	260	130
7	350	60	110	1,680	490	150	130	120	890	9,320	350	3,720	290	810	24,610
8	430	110	1,550	230	1,140	260	80	2,880	330	22,050	470	110	1,930	2,100	460
9	260	90	1,140	1,080	1,050	580	370	1,490	150	2,190	110	5,490	3,330	2,820	990
10	570	950	4,740	510	2,400	410	1,050	3,500	8,460	2,300	390	3,100	5,990	1,210	1,430
11	4,080	6,920	370	590	510	3,600	14,000	2,640	5,990	2,210	6,800	3,860	8,600	4,900	1,750
12	4,140	370	270	280	380	3,180	220	270	260	340	3,500	1,200	7,800	2,340	1,220
13	290	110	90	190	530	210	90	310	26,440	210	170	2,280	1,610	4,750	270
14	190	160	210	140	70	160	40	80	140	410	390	580	460	380	260
15	60	60	110	90	70	190	60	210	80	100	220	70	2,460	120	490
16	50	30	60	290	50	40	20	110	290	110	60	30	10	250	70
Total	30,620	15,880	41,900	49,830	26,610	20,160	42,140	74,600	83,130	92,960	21,440	33,560	43,700	57,740	66,780
Average	1,914	992	2,619	3,114	1,663	1,260	2,634	4,662	5,196	5,810	1,340	2,077	2,731	3,609	4,174

other the milk should be strained. The strainer on the milking-pail answers this purpose, and it seems advantageous to retain this strainer but to omit the second straining through cotton.

In this series of experiments we also find a great variation of numbers of bacteria in milk, as the table shows. The numbers decrease with the decline of temperature. Occasionally abnormally large numbers of bacteria were found. This is probably due, among other circumstances, to changes in the force of milkers, and possibly also to the fact that some cows average higher in bacteria than others.

Several tests were made from the liquid squeezed out from the cotton strainer after all milk had passed through. The counts show that this liquid teems with bacteria, varying from 50,000 to 100,000 per c.c. This fact tends to confirm our conclusion in reference to the effect of cotton strainers.

TABLE 6.

TOTALS AND AVERAGES OF BACTERIA IN MILK BEFORE STRAINING THROUGH COTTON, ON TOP OF STRAINER, AND AFTER STRAINING

Milk before Straining	Milk on Top of Strainer	Milk after Straining
1,014	1,260	1,340
992	2,634	2,097
2,610	4,662	2,731
3,114	5,196	3,609
1,663	5,810	4,174
Total....... 10,302	19,562	13,951
Average.... 2,060	3,912	2,790

AN ATTEMPT AT CLASSIFICATION OF BODY CELLS IN THE SLIME OF THE SEPARATOR.[1]

A limited study of the relative number and character of the white corpuscles and the bacteria present in separator slime was carried on in connection with the bacteriological work. For this purpose thin films of the slime were prepared daily for 16 consecutive days and half the number of these films fixt with alcohol, the other half with heat. Other films fixt by heat (made by passing the slide through the flame) were stained immediately with Löffler's methylene blue and examined. The remaining preparations were properly dated and taken to the laboratory at Chicago for a more careful examination. The films fixt with alcohol were stained with Giemsa's stain; the films fixt by heat were stained with methylene blue, and in several

[1] This part has been worked out and written by A. B. Luckhardt.

instances with hematoxylin and eosin, and some also with Weigert's fibrin stain.

In general the preparations revealed numerous polymorphonuclear leukocytes, occasionally containing many cocci or short chains of streptococci. Bacilli were rarely met with. In addition to the polymorphonuclear leukocytes there were seen small lymphocytes, appreciable numbers of large mononuclear leukocytes, as well as large cells of irregular outline with greatly vacuolated protoplasm and very pale nucleus. These latter cells were often phagocytic in nature, which fact lends support to the belief that they represent desquamated epithelial cells. As many as 20 cocci, usually diplococci, were observed in a single one of these cells. Fibrin threads were frequently present and were positively identified by means of Weigert's fibrin stain. On three consecutive days and after six days out of the 16 days, on which smears were made, the preparations stained with Giemsa's stain revealed a great number of typical eosinophiles. The protoplasm of these cells was usually filled to so large an extent with the sharply circumscribed eosinophilic granules that the nucleus was hidden from view. The cells varied in size, some being quite large and others small. Some were spherical, some ovoid. The nucleus was sometimes single, usually multilobed or distinctly polynuclear. On one of the slides the granules in the cell were plainly visible in the methylene blue stain. In this case, however, the granules had a brassy tint and not a distinct red coloration as observed in the differential Giemsa preparations.

The paucity of bacteria in all preparations surprised us and suggested the possibility that the dried plasma and the slimy sediment protected the bacteria from the action of the stain. The great number of bacteria in the separator slime of milk from a neighboring dairy and the ease of their detection convinced us that the difference in bacterial numbers per field of the microscope between the two preparations represented to some extent the difference in cleanliness in the production of milk in the two dairies.

In an exhaustive critical review of the literature on "The Relative Importance of Streptococci and Leukocytes in Milk" Harris[1] has pointed out that the "unfortunate choice of the term 'pus cell' instead

[1] *Jour. Infect. Dis.*, 1907, Supplement No. 3, p. 50.

of leukocyte is to be regretted." According to the classification proposed by Ehrlich and adopted by most hematologists today the white corpuscles occurring in blood belong to one of two classes, the lymphocytes and the leukocytes. The leukocytes are furthermore subdivided according to their morphology into mononuclear leukocytes, polynuclear leukocytes with neutrophile granules, or with eosinophile granules (eosinophiles), and mast cells. When speaking of pus it is most generally understood that there occur, aside from cell detritus, bacteria, fibrin, large numbers of polymorphonuclear leukocytes containing neutrophile granules. The latter are the pus cells par excellence. As far as we are aware, no investigator of the white corpuscles in milk has attempted to differentiate between them. There are no definite statements in the literature whether or not the so-called "pus cells" or leukocytes of other authors are the polymorphonuclear leukocytes of the pathologist. Our rather limited investigation, which is being completed, shows that milk of unquestionably healthy cows contains large mononuclears, small lymphocytes, and in some instances eosinophiles in appreciable numbers, altho these are not so numerous as polymorphonuclear leukocytes of the neutrophile type. In general the appearance of eosinophiles in the tissues and blood of animals, aside from cases of myelogenous leukemia, is an indication of infection of parasitic etiology, i. e., trichinosis in man, scleromiasis in horses, uncinariasis in man.

The importance or significance of eosinophiles in milk in particular is as obscure as the significance of the presence of white corpuscles in milk in general. Doane[1] writes: "There seems to be but one conclusion, which is that, either in the elaboration of the milk in the udder, or through the intimate association of the blood vessels in the glands, leukocytes escape into the milk." This is in accordance with the statement of Ellenberger and Günther[2] that the appearance of numerous leukocytes in the secreting alveoli of the mammary gland at the beginning (colostrum milk) and toward the end of lactation, signal the beginning and the end of lactation, and that under normal physiological conditions the number of leukocytes found in the alveoli decreases until final disappearance during active

[1] *Bull. No. 102, Maryland Agric. Exp. Sta.*, 1905.

[2] *Grundriss der vergleichenden Histologie der Haussäugethiere*, 1908, pp. 205, 206.

lactation. Russell and Hoffmann[1] "noted frequently a high cell content in the earlier as well as in the later stages of the period of lactation." Ellenberger and Günther furthermore state that, even in the active gland, not all the alveoli are in a state of activity, but that some alveoli and groups of alveoli, which are in a resting stage, contain numerous leukocytes in the connective tissue of the stroma of the gland and sometimes between the cells of the alveoli themselves, so that their subsequent extrusion into the alveoli and appearance in the milk are made very probable. These observations partly account for the wide variation in count of the white corpuscles in different healthy cows as noted by Bergey,[2] Doane,[3] Savage,[4] and Russell and Hoffmann.[5]

Harris suggested that "apart from any signs of inflammation, chemiotactic substances may play a part in leukocytosis, due to the residence of saprophytic streptococci within the udder." If, aside from these possible factors, the appearance of white corpuscles is due to the intimate association of the blood vessels with the glands, as Doane suggested, this anatomical arrangement would likewise explain the appearance of the great number of eosinophiles which are occasionally met with, for about 13.15 per cent of the white corpuscles of normal cow's blood consists of eosinophiles (Dimock and Thompson, quoted by Burnett[6]).

It appears to us that the morphology of the various white corpuscles of cow's milk and blood under normal and pathological conditions ought to receive careful investigation, just as has the morphology of the white corpuscles of the blood of healthy and diseased animals. Such a study will no doubt lead to a more satisfactory classification of the white corpuscles found in milk and will place subsequent investigations in this field on a proper scientific basis and forever render inexcusable the loose use of the term "pus cells," which suggests suppurative processes, and the general term leukocyte as including all white corpuscles present in milk.[7]

[1] *Report Wis. Agri. Exp. Sta.*, 1907, p. 231.

[2] *Bull. No. 125, Dept. of Agri., Commonwlth. of Pennsylv.*, 1904.

[3] *Bull. No. 102, Maryland Agri. Exp. Sta.*, 1905.

[4] *Jour. of Hyg.*, 1906, 6, p. 123.

[5] *Loc. cit.*

[6] *Clinical Pathology of the Blood of Animals*, 1908, p. 43.

[7] Since this paper has gone to the printer, there has come to hand a paper by Hewlett, Villar, and Rives in the *Jour. of Hyg.*, 1909, 9, p. 271, "On the Nature of the Cellular Elements Present in Milk."

SUMMARY AND CONCLUSIONS.

1. Separator cream contains a smaller number of bacteria per c.c. than the whole milk from which it is obtained. Separator milk contains more bacteria than the whole milk from which it is obtained.

2. The bacterial count in milk obtained by mixing the cream and skim milk from the separator is higher than that of the original milk.

3. Straining milk, during the process of milking, through a brass sieve removes some of the bacteria, and also removes coarse particles of dirt, which otherwise would increase the number of bacteria.

4. Straining milk through absorbent cotton before bottling results in a higher bacterial count for the strained milk.

5. Polymorphonuclear leukocytes of the neutrophile type, large mononuclear leukocytes, and small lymphocytes appear normally in the separator slime of the milk of healthy cows, and as far as we can see they bear no relation to the number of microorganisms present, inclusive of streptococci.

6. Eosinophiles may occur in the slime of the separator. The cause and significance of their presence remain problematical.

7. The white corpuscles in milk of normal and diseased cows and in the blood of the same animals should be studied, differentiated, and classified. Such a study will put the subject of leukocytes in milk on a more exact scientific basis than heretofore, and further our knowledge of the significance of the relative number of the various corpuscles in milk in normal and diseased conditions of the cow in general, and in pathological processes of the mammary glands and the udder in particular.

The authors believe that the cellular elements constantly found in milk and described as polymorphonuclear leukocytes are to be looked upon as "detached young epithelial cells" which arise from the germinal layer and have been stimulated to great multiplication by the invasion of streptococci into the lumina of the alveoli. The authors also report the histological appearance of the mammary glands as described by Pfaundler in various stages of glandular activity. We quote part of the description of one of the stages: "The cells appear indefinite, their outline and limiting surfaces indistinct. There is a marked infiltration of the alveoli with leukocytes (many eosinophiles), which are found in such great numbers in the interstitial tissue and the epithelial layer and lumina of the alveoli, that the remaining structure is only recognized with difficulty." We were unaware of this fact when the article was written. The great infiltration of the glandular tissue by the eosinophiles and their subsequent extrusion into the alveoli probably explain our detection of them in the separator slime. We are of the opinion that the majority of the cellular elements found in the separator slime are white blood corpuscles, particularly polymorphonuclear leukocytes. On the other hand, we are ready to admit that some of the elements are distinctly desquamated epithelial elements. The authors also record the practical absence of phagocytosis. Our observations on this point, altho not extensive, revealed many examples of phagocytosis, both by the pholymorphonuclear leukocytes and by desquamated epithelial cells.

DAIRY SCORE CARDS.

P. G. Heinemann.

Dairy score cards for the guidance of inspectors are gaining in favor rapidly. Several plans have been proposed, the principle of which is the enumeration of the conditions to be considered and criticized. By rating these conditions with a certain number of points according to merit, the relative degree of perfection is determined. The sum of the points for perfect scores varies in different cards from 100 to 500. The score card which is probably the most popular has been designed in the Dairy Division of the Department of Agriculture in Washington. This card allows 40 points for a perfect score in equipment, and 60 points for methods, a total of 100 points for a perfect dairy. It is desirable to have uniform methods of inspection throughout the country, so as to give comparable results, and the figure 100 for a perfect score is perhaps the most practical. If the number is much higher it is rather difficult to distribute the points proportionately to the relative importance of the various items; on the other hand, if the number is too low, it is difficult to go into detail to a degree necessary for proper guidance of the inspector. During the writer's experience in scoring dairies the card designed by the Department of Agriculture has been used, and altho excellent results have been obtained, it seemed profitable to extend some of the items and give more detail. This is important in view of the fact that the development of dairy conditions is progressing rapidly and new points are constantly coming up which must be represented on the score card. The card as it now stands is not quite specific enough in some instances and consequently leaves too much to the judgment of inspectors who are sometimes inexperienced and perhaps prejudiced. One of the chief benefits of the score card system of inspection is that suggestions are given to inspectors who are not accustomed to this kind of work, and even in the hands of experts the card is a good reminder, not only of points which may otherwise be overlooked, but particularly of points which previously have been found to be amiss. In a modified score card the writer has made an attempt to overcome the shortcomings pointed out, and in order to carry out this idea it was found necessary to increase the total number of points to 200. The final score is then divided by two

DAIRY SCORE CARD.

EQUIPMENT.

		Score: Perfect	Score: Allowed
COWS			
Health		8	....
Apparently in good health	1		
Tuberculin tested once a year, reacting animals found and removed	1		
Tuberculin tested twice a year, reacting animals found and removed	3		
Tuberculin tested once a year, no reacting animals found	5		
Tuberculin tested twice a year, no reacting animals found	7		
Comfort		6	....
Temperature of stable	1		
Bedding renewed daily	1		
Kind of bedding	4		
Hay	1		
Clean straw	2		
Wood shavings	4		
STABLES			
Location		3	....
Construction		14	....
Loft	3		
Holes in ceiling tightly shut	1		
No holes, food brought in from without	2		
Loft not used or absent	3		
Floor	3		
Tight wood	1		
Cement	2		
Cement covered with wood in stalls	3		
Gutter	2		
Tight wood	1		
Cement	2		
Walls tight	1		
Ceiling tight	1		
Stalls	2		
Clean wood partitions	1		
Iron pipe partitions	2		
Stanchions	2		
Movable wood stanchions	1		
Swinging iron pipe stanchions	2		
Drainage		2	....
Light		5	....
Distribution	1		
Amount	4		
3 square feet per cow	1		
3½ square feet per cow	3		
4 square feet per cow	4		
Ventilation		4	....
Cubic space per cow		4	....
Less than 300 cubic feet	0		
300–400 cubic feet	1		
400–500 cubic feet	2		
500–600 cubic feet	3		
600–800 cubic feet	4		
800–1,000 cubic feet	3		
Above 1,000 cubic feet	0		
Washstands in stable		1	....
Stable for sick cows		3	....
Construction	1		
Condition	1		
Removed 100 feet or more from other stables	1		
MILK ROOM			
Location		2	....
Convenient	1		
Clean surroundings	1		
Construction		3	....
Walls and ceiling tight	1		
Walls cemented	2		
Walls enameled	3		
Light		2	....
Abundant	1		
Distribution	1		
Ventilation		1	....
Doors and windows screened		1	....
Floor		2	
Cement	1		
Tile or other smooth material	2		
Reservoir for milk outside of milk room		2	....
If protected	2		
Not protected	1		
MILKING-SUITS			
White suits and caps		1	....
Care of suits		2	....
One clean suit daily	1		
Two clean suits daily	2		
UTENSILS			
Small top milk pail		3	....
Cans seamless		1	....
Bottles capped by machine		1	....
Caps sterilized		1	....
Facilities for washing and sterilizing bottles		2	....
MILK COOLER			
Covered		1	....
Cooling		3	....
Cooled with well water	1		
Cooled with ice water	2		
Cooled with brine	3		
FOOD			
Food clean and wholesome		2	....
Total		80	....
METHODS			
COWS			
General condition		5	....
Special conditions		5	....
Udders clipped	2		
Soiled hair on body kept clipped	1		
Legs curried and kept clean	1		
Tails washed and kept clean	1		
STABLES			
Cleanliness		16	....
Floors	4		
Cleanliness	2		
Free from hay, etc.	2		

Score for Equipment.....+Score for Methods......=......Divided by 2......=......Final Score.

DAIRY SCORE CARD.

Methods.

		Score: Perfect	Score: Allowed
Walls clean and free from cobwebs	2		
Ceiling clean and free from cobwebs	2		
Ledges clean	2		
Mangers clean	2		
Partitions and iron pipe clean	2		
Gutters clean	2		
Windows and doors		4	....
Cleanliness	2		
Screened	2		
Ventilation		2	....
Stable air		6	....
Good in general	3		
Sweet at milking time	3		
Removal of manure from stable		4	....
Daily by shovel	1		
Daily by wagon	2		
Daily by wheelbarrow	3		
Daily by suspended carrier	4		
BARNYARD			
General condition		4	....
Drainage	2		
Cleanliness	2		
Removal of manure from barnyard		3	....
To pit 50 feet distant	1		
To pit 100 feet distant	2		
To pit more than 100 feet distant	3		
FOOD			
Perfect score for food		4	....
Handling of food	2		
Silage fed after milking only	1		
No food shortly before milking	1		
BOTTLING-ROOM			
Cleanliness		2	....
Hot water for cleaning	1		
Live steam for cleaning	2		
Room steamed before bottling		1	....
Condition of windows		2	....
Cleanliness	1		
Screened	1		
Milk covered during passage to cooler		4	....
UTENSILS			
Washing of bottles		2	....
In boiling water	1		
In live steam	2		
Handling of bottles		2	....
Kept inverted	1		
Kept inverted and placed in bottling room hot	2		
Cans and pails		8	....
Cleanliness	5		
Washed, not scalded	1		
Washed and scalded	3		
Washed and steamed	4		
Washed and steamed under pressure	5		
Inverted in pure air	3		
MILKING			
Clean dry hands		3	....
Hands washed for each cow		1	....
Milk weighed in separate room		2	....
Udders washed before milking		3	....
First streams of milk removed		2	....
Spilled on floor	1		
Collected in separate vessel	2		
Cleanliness of milkers		2	....
Milking stools		2	....
Clean wood	1		
Clean iron	2		
Milk pail		2	....
Strainer clean	1		
Strainer sterilized	2		
HANDLING OF MILK			
Cleanliness of attendants		2	....
Milk promptly removed from stable		2	....
Cooling of milk		8	....
Below 60° F	1		
Below 55° F	2		
Below 50° F	4		
Below 45° F	6		
Below 40° F	8		
Time elapsing between milking and cooling		5	....
30 minutes	1		
25 minutes	2		
20 minutes	3		
15 minutes	4		
10 minutes	5		
Storage		8	....
Below 60° F	1		
Below 55° F	2		
Below 50° F	4		
Below 45° F	6		
Below 40° F	8		
Transportation		4	....
Dry blankets or covered wagon	1		
Jacket or wet blanket	2		
In boxes filled with chipped ice	4		
Total for Methods		120	....

Score for Equipment......+Score for Methods .....=......Divided by 2......=......Final Score.

(*Reverse of card*)

DAIRY SCORE CARD.

Owner or lessee of farm..

P. O. address.......................................State..............................

Total number of cows.......................Number milking....................

Date of inspection.., 190....

Remarks...

....

....

....

(*Signed*)..

Inspector

and by this method the score is again on the basis of 100, thus making the results comparable to the results obtained with the Department card. The general plan of the Department card has been retained and taken as a basis for making extensions and additions. Producers of milk are gradually being educated to understand the principles involved in sanitary milk production and are introducing improvements in their dairies in many ways. In justice to the most progressive ones the score card should be designed to record these improvements in some manner, which object can be obtained only by stating details.

It is hardly profitable to discuss the various points where changes and additions have been made. It may be stated, in a general way, that an effort has been made in designing this score card to give sufficient detail for the guidance of the inspector and to remind him of the conditions to be scrutinized and criticized without omitting any necessary parts. This card has been of great assistance in recalling certain points which at previous inspections had to be criticized. It is an advantage also of this card that it is never necessary to use fractions of a point. The card permits more delicate grading than previous cards, without giving occasion for favoring one dairy more than another. Slight, but more or less important, differences in equipment and methods employed are recorded with accuracy and this fact may act as a stimulant to the dairies concerned to make improvements more rapidly.

A score card which states details without being overburdened may also have the advantage of enabling boards of health, milk commissions, large dairy concerns, etc., to classify the dairies accord-

ing to their equipment and management and thus control the output. The score of a dairy producing certified milk, for instance, might range from 95 to 100, of a dairy producing inspected milk 85 to 95, etc. The exact range must be decided upon by the supervising body. The progressive improvements are naturally shown to better advantage in proportion to the sensitiveness of the score card, and inasmuch as the plan of the card, here presented, is substantially the same as the plan of the Department of Agriculture card, and inasmuch as the same numerical relation is obtained by dividing the result by two, the figures remain comparable whether obtained by one card or the other.

THE REACTION OF VARIOUS BACTERIA UPON AESCULIN AGAR.*

OSKAR KLOTZ AND A. C. RANKIN.

(From the Pathological Laboratory of the Royal Victoria Hospital, Montreal.)

GREAT attention has been given to the bacteriological analyses of "open waters" to determine their value as potable waters. Exhaustive determinations have been made of the bacterial flora of various rivers and lakes. The common result of most of these observations has been that the colon bacillus is present to a greater or lesser degree in every "open water." However, tho different streams may contain colon bacilli, it is found that the numbers which are present in a definite quantity of the water vary; but when a certain stream is analyzed at intermittent periods for a year or more, it is found that the number of bacilli present in that water is fairly constant for the same period in the year, except when a new source of pollution has entered the stream within the viable distance of the colon bacillus. If this source of pollution is continuous, a new colon standard is developed for that stream.

It is universally accepted that B. coli is a fair indicator of pollution in any water, and that when the "normal" coli content has been determined in a water, any unusual fluctuation, arising above this normal, is indicative of a new pollution.

Hence, too, a bacteriological analysis of water mainly concerns itself in the determination of the presence of colon bacilli, besides estimating the total bacterial count per cubic centimeter of water, and the number of liquefiers of gelatin present. When occasion demands, definite pathogenic organisms are searched for in the water.

As, therefore, the routine water analysis is so closely associated with colon bacilli, many investigators have devised special methods to determine the colon bacillus in definite quantities of water. Most of these methods have been, in truth, devices for separating the colon bacillus from the many other organisms present in the water, and some workers have obtained a definite biological reaction on certain media to serve as an indicator of the presence of this bacillus.

* Received for publication October 5, 1909.

It has been found that the colon bacillus is more resistant to certain chemical substances than are other bacteria, so that media containing these substances will propagate this bacillus better than others. Such media have been prepared with phenol and hydrochloric acid (Parietti), crystal violet (Conradi-Drigalski), malachite green (Löffler), bile (McConkey), caffein, and other substances. By the use of these media most of the bacteria other than those belonging to the colon-typhoid are group inhibited in their growth. From such cultures then the remaining bacteria may be isolated and their characters determined. Some workers deem it quite sufficient to inoculate dextrose fermentation tubes from growths in the above inhibitory media, and to determine (by the production or non-production of gas in the proportion of $H:CO_2 = 2:1$) the presence or absence of the colon group. This presumptive test is used extensively in America. In the English laboratories the use of the bile-salt media has found preference (McConkey).

It is scarcely necessary to point out that most of the presumptive tests have their shortcomings in one way or another. We have been particularly struck with the inaccuracy of the gas determination for the colon bacillus. We have noted that when colon bacilli were present in mixt cultures in the carbohydrate broth either they produced no gas whatever, or that the amount of gas was very much reduced, and in altered proportions.

Recently Harrison and van der Leck[1] have introduced still another medium for the determination of colon bacilli and excretal bacteria in water or milk. They have found that when the glucoside (aesculin) is present with iron citrate in the culture medium, the colonies of colon bacilli are black and have a black halo around them. These black colonies are readily distinguishable against a white background. They have found that B. lactis aerogenes, lactose-fermenting yeasts, and some molds give the same blackening of the media about the colonies as do colon bacilli. As, however, the yeasts and molds are readily distinguished in their colonies and are seldom found in water, they may be disregarded. In some of the experiments Harrison and van der Leck found that the colonies of B. lactis aerogenes could be distinguished from those of the colon bacilli. They point out

[1] *Centralbl. f. Bakt.*, Abt. II, 1909, 22, p. 547; *Proc. Roy. Soc. Can.*, 1908, S. III, 2, p. 105.

that as the B. lactis aerogenes may be regarded as an excretal form of organism, it is of value to recognize its presence. The authors state that some 40 species or varieties of bacteria and yeasts have been grown in media containing aesculin with negative results.

The authors have prepared a bile-salt aesculin broth and an aesculin agar which they recommend for the routine examination of water. In using the former medium the presence of colon bacilli is indicated by the black coloration of the fluid, and they consider this a better presumptive test than the use of the neutral red bile-salt broth.

The authors have found that in the analysis of water, the blackened bile-salt aesculin broth has invariably yielded colon bacilli. In the analyses of 60 samples of water, they have found the aesculin bile-salt test correct in 100 per cent.

In the following experiments we have undertaken the examination of many strains of colon bacilli, which have been isolated and fully determined as to their characters, besides testing the action of other bacteria on the aesculin media. Most of the bacteria tested were obtained from patients in the Royal Victoria Hospital. These bacteria had been isolated from time to time, and were retested as to their purity on several occasions.

In determining the action of the bacteria on aesculin media, we have used the formula as given by Harrison and van der Leck. The method of preparing the agar is as follows:

1 or 2 gm. of Witte's peptone.
0.5 gm. sodium taurocholate (commercial).
0.1 gm. aesculin.
0.05 gm. iron citrate (ferric).
100 c.c. tap water.

After steaming 15 to 30 minutes the medium is filtered and sterilized. For aesculin agar 1.5 per cent agar is used, being dissolved in part of the water, after which the other ingredients are added, the medium brought to boiling, filtered, tubed, and sterilized.

Plates were poured with this medium, and after hardening, the different organisms were planted on the surface, or deep colonies were made. The plates were incubated at 37° C. and were examined every 24 hours for a period of two weeks.

In most instances in which a positive result was obtained, the dis-

coloration of the medium occurred within 24 hours. In a few instances, however, the positive result was delayed for several days. One of the colon strains gave a positive result on the second day. Another colon strain was positive only on the third day, while a strain of B. mucosus remained negative until the fifth day, when a slight blackening of the medium occurred, which increased for several days. We have found that there is a certain variation in the positive tests. The typical positive tests in which the colonies are black with a black halo around them, as described by Harrison and van der Leck, do not constantly occur. Four colon strains we found gave only a faint brownish discoloration of the medium, immediately about the colony, while the colony itself was of a greyish-brown color. Again other colon colonies gave a reddish-brown discoloration of the medium without definite blackening of either the colony or the medium. One strain of proteus, which gave a positive result, first developed a yellow change in the medium, which later showed a greyish halo around the outer edge of the discoloration.

On account of the variable types of the color reactions which were obtained on the medium, we have included in the "positive tests" all reactions which in any way discolored the medium. This discoloration ranged, as above noted, from a yellowish-brown to a distinct black. The tests referred to as negative were those in which no color was present in either the colony or the medium. In this way we have tested 110 organisms of different strains, and from various sources with the following result:

	Positive Test on Aesculin Agar	Negative Test on Aesculin Agar		Positive Test on Aesculin Agar	Negative Test on Aesculin Agar
B. coli communior	3	10	B. iliacus	1	2
B. coli communis	10	6	B. cloacae	1	4
B. typhosus	0	19	B. subcloacae	1	0
B. typhosus-like organism	1	0	B. proteus vulgaris	1	9
B. paratyphosus B	0	13	B. proteus plebeius (Ford)	0	1
B. mucosus	3	1	B. pylori (Ford)	0	1
B. duodenale (Ford)	1	2	B. xerosis	0	1
B. lactis aerogenes	3	1	B. subtilis	0	1
B. pseudodysenteriae (Muller)	1	1	B. diphtheriae	0	3
B. dysenteriae (Flexner)	0	1	Staph. aureus	0	2
B. acidiformans	0	1	Staph. albus	0	3
B. alkaligenes	0	2			

It will be seen from the above table that most variable results were obtained from the various organisms. B. coli communior gave only

three positive tests in 13 strains, while B. coli communis gave 10 positive reactions in 16 strains. The positive tests obtained with the B. coli communis and communior were far from being uniform, and the color reactions varied from those producing no color at all, others producing a faint greyish-brown, to those showing red-brown, brown, or black color. These color reactions were evident either in the colonies themselves, or in the surrounding medium, or in both. The majority of the colon strains which gave a color reaction showed the dark punctate center in the colony with a black halo surrounding the colony, as described by Harrison and van der Leck.

Among our stock of 19 typhoid strains none was found to give any color reaction on aesculin agar. We have, however, an organism which was isolated from the gall-bladder of a typhoid subject, and which on media gave all the reactions of B. typhosus. At the time of isolation, this organism was tested with a known typhoid serum but gave a negative result. Subsequently at intervals of about a month this organism has been tested with various typhoid sera and has never agglutinated in dilutions higher than 1.25. Except for the agglutination reaction, this bacillus has all the features of B. typhosus. This bacillus was found to give a positive reaction on aesculin and the reaction was positive only on the seventh day, no reaction showing in the six days previously. The medium about the colony was discolored a faint brown which increased to a darker brown after several days. The reaction here obtained, altho not typically positive, as described by Harrison and van der Leck for certain colon strains, was nevertheless equal in intensity to that obtained with several colon organisms tested by us, and noted as positive in the above table.

Again, among four B. lactis aerogenes strains, we had one which remained negative in its reaction on aesculin agar after 14 days.

Summarizing our results, we found that in 62 different strains of 13 types of intestinal bacteria of man, 22 gave a positive reaction on an aesculin, while 40 were negative. In other words, the majority of the excretal type of bacteria, which were examined by us, and which had their known origin from man, gave a negative result when tested on the above medium.

In our hands this medium has not proved satisfactory for the

isolation and determination of B. coli and other human excretal bacteria.

NOTE.—Since going to press Harrison and van der Leck have in a recent article (*Centralbl. f. Bact.*, 1909, 51, p. 607) described some modification in the preparation of the media. The amount of iron citrate has been increased to 0.1 per cent, and they insist that the acidity of the media should be 0.6 per cent acid. The media which we have used in our experiments was of this acidity, but contained a smaller quantity of iron citrate. The authors point out that with an acidity lower than 0.6 per cent the results are not uniform. It is found too that the color of the colonies is dependent directly upon the amount of citrate and acid present.

A NEW COLOR MEDIUM FOR THE ISOLATION AND DIFFERENTIATION OF STREPTOCOCCI.*

D. D. Todd.

(From the Memorial Institute for Infectious Diseases, Chicago.)

It is the purpose of this paper to give briefly the results of tests of the action of various strains of streptococci on various dyes in sugar media. The work was commenced on the assumption that some definite color reaction might be found characteristic of different strains of the cocci depending on the action of the products of fermentation on the dyes.

Previously but little has been said on this basis of differentiation of streptococci, altho the principle involved is a common one, as seen in the various color media for the isolation of B. typhosus and other bacteria. A plate method of great importance is that of Schottmüller[1] in which, by the use of blood-agar, he differentiated Strept. pyogenes, Strept. viridans, and Strept. mucosus. This method, in reality, represents two properties of the cocci, the hemolytic power of Strept. pyogenes and the fermentative power of Strept. viridans, for Ruediger[2] has shown that the green coloration of the latter is probably due to the action of the coccus on the muscle sugar in the agar.

Gordon[3] experimented with a watery solution of neutral red which he added to broth as a means of differentiation. He found, in anaerobic cultures, that Strept. brevis (Lingelsheim) reduced the dye with the production of a green fluorescence, while Strept. longus (Lingelsheim) and Strept. pyogenes reacted negatively. Fregonneau[4] tested methyl orange and found no characteristic color reaction for numerous bacteria, including the streptococcus.

In carrying out my tests, about an equal number of strains of Strept. pyogenes and Strept. viridans (Schottmüller) were isolated from the

* Received for publication October 4, 1909.

[1] *Münch. med. Wchnschr.*, 1903, 50, p. 909.

[2] *Jour. Infect. Dis.*, 1906, 3, p. 663.

[3] *Centralbl. f. Bakt.*, Orig., 1904, 35, p. 271.

[4] *Ibid.*, Orig., 1909, 49, p. 276.

mouth by the use of the blood-agar plate method.[1] Each strain was inoculated in a double set of media, and only those were selected which corresponded in growth in both sets. A working classification was made by taking the degree of fermentative action in litmus milk (see Table 1). This gave four groups, and the numerical frequency with which the other cultural characteristics agreed placed the strains in different sub-groups. A number of the strains of each

TABLE 1.
CLASSIFICATION OF STREPTOCOCCI.

Milk Culture	Number of Strains	Gram Positive	Morphology			Colonies in Blood-Agar Plates		Broth Culture			Gelatin	
			Elongated		Round			Turbid	Clear			
			Long Chains	Short Chains	In Chains	Hemolysis	Green	Uniform	Diffusible	Flocculent	Growth	No Growth
No coagulation												
Slight acidity	12	12	4	1	7	10	2		11	1	4	8
Marked acidity	23	23	8	11	4	19	4	3	14	6	12	11
Coagulation												
Deep red	26	26	7	14	5	12	13	10	7	7	19	7
Complete decolorization	13	13	1	11	1	11	2	4	6	3	11	2

sub-group were tested on the dye sugar media. No attempt was made to apply Gordon's tests[2] or to arrange the cocci according to the grouping of Andrewes and Horder,[3] it being the desire to make the classification of the cocci, based on their fermentative powers, as simple as possible. This was necessary, if a plate method of differentiation was to be found in which only one sugar could be used and that necessarily being one commonly fermented by all strains of streptococci.

The media were made as usual. A large amount of sugar-free agar was prepared so as to have a uniform medium on which to make the tests. To this two per cent sugar was added. The sugars were dextrose, lactose, and saccharose, which are fermented by all strains of streptococci. Later it was found that any one of the sugars gave the same reaction with dyes and accordingly lactose was used exclusively.

[1] The strains of *Strept. viridans* were grown on inulin agar, in addition to the morphological and cultural tests, to differentiate them from the pneumococcus according to the method of Ruediger, *Jour. Infect. Dis.*, 1906, 3, p. 183.

[2] *Lancet*, 1905, 2, p. 1400.

[3] *Ibid.*, 1906, 2, pp. 708, 775, 852.

As to the amount of dye added, considerable experimentation was required. Rothberger[1] reported the use of a large number of dyes in liquid media in an attempt to differentiate various bacteria. The amount of dye he added was just a sufficient number of drops of saturated watery solution of the dye to color the broth. This idea was used as a guide to the first determination of the approximate amount of dye to add to the sugar medium. A one per cent solution of the dyes was accurately made and measured amounts of this were added to a definite volume of liquid agar to just give a trace of the characteristic color of the dye to the medium. Several strains of the streptococci

TABLE 2.
DYES (GRÜBLER) TESTED IN AGAR MEDIUM.

Acid Stains	c.c. per Liter*	Basic Stains	c.c. per Liter*
Congorot	5.0	Bismarck braun	10.0
Corallin	3.0	Chrysoidin	3.0
Eosin	3.0	Dahlia	2.0
Indigcarmin	3.0	Fuchsin	2.0
Indulin	3.0	Janusgrün†	2.0
Metanilgelb	5.0	Jod grün	2.0
Nigroisin	5.0	Magdalaroth	5.0
Rubin S	5.0	Magentaroth	2.0
Säurefuchsin	5.0	Malachitgrün†	2.0
Säureviolett	5.0	Methylengrün†	2.0
Wasserblau	10.0	Methylenblau†	0.2
		Neutralroth	0.4
		Phloxinroth	3.0
		Safranin	3.0
		Toluidinblau	3.0

* One per cent solution of dye. † Pneumococci failed to grow.

were then plated out in each dye medium, and the amount of dye per liter of agar, in which a good growth of streptococci was obtained, is shown in Table 2. The medium was sterilized for three successive days in the Arnold sterilizer. Sterile beef serum to the amount of 2 c.c. was added to each tube of media (7–8 c.c.) just before plating. This was found to make the growth of the cocci more rapid, and to add to the characteristic appearance of the colonies by the formation of a milky-like haze around the colonies, which is probably due, as explained by v. Hecht and Hulles,[2] to the formation of an albuminous precipitate as the result of the acid formation from the action of the cocci on the sugar.

After testing various dyes, three were found which gave a definite but a similar color colony with the different strains of streptococci,

[1] *Centralbl. f. Bakt.*, Orig., 25, pp. 15, 72.

[2] *Ztschr. f. Hyg.*, 1909, 63, p. 113.

the dyes being neutral red, rubin S, and acid fuschin. As the three dyes gave similar color colonies, I confined myself to the use of the neutral red and in lactose agar medium.

The different groups of streptococci (Table 1) were repeatedly tested in the neutral red lactose agar medium and only two distinct color colonies were found. On classification of the strains according to these color colonies, the latter were found to represent exactly Strept. pyogenes and Strept. viridans (Schottmüller), or the two types of strains originally isolated. It thus appeared that in the use of the neutral red lactose agar medium lay a method of isolation and differentiation of Strept. pyogenes and Strept. viridans. It further suggested its possible use for the differentiation of allied species.

A number of strains of Strept. mucosus were isolated and tested on the color medium and found to produce a distinct color colony. Five strains of pneumococcus[1] were tested and found to produce color colonies that could be differentiated from the streptococcus.

The deep colonies were not definitely characteristic; the surface colonies appeared as follows:

Strept. pyogenes.—Colonies 1 mm. or less in size, deep red in color with a barely perceptible pink border, margin smooth and sharply defined. After 18–24 hours a slight milky-like haze surrounds the colony, and irregularly spreads from the colony with further incubation.

Strept. viridans (Schottmüller).—Colonies 1–3 mm. in size, red center with a pink border $\frac{1}{5}$–$\frac{1}{3}$ width of center, margin smooth with surrounding large area of milky-like haze. With over 50 colonies the haze or opacity extends entirely over the plate.

Strept. mucosus (Schottmüller).—Colonies 4–5 mm. in size, pink center with white border equal to width of center or greater, margin very irregular and serrated, slight surrounding milky-like haze which increases slowly with incubation.

Pneumococcus.—Colonies 2–3 mm. in size, red center with a narrow white border, margin smooth and sharply defined, slight surrounding milky-like haze which increases but little with incubation.

I have repeatedly isolated the above types of cocci, according

[1] Kindly given by Dr. E. C. Rosenow.

to the description of the colonies given, using the neutral red lactose medium to which has been added the beef serum. My work has been confined solely to the bacteria of the mouth, for which, at least, I hold the medium to be of some value for the isolation and differentiation of streptococci and pneumococci.

METHODS FOR TESTING SHELLFISH FOR POLLUTION.*†

Stephen DeM. Gage.

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In March, 1901, acts were passed by the legislature of Massachusetts, directing the State Board of Health to examine annually the conditions about the main sewer outlets of cities and towns and report thereon, and prohibiting the taking of oysters, clams, quahogs, and scallops from polluted sources. Acting under this authority, investigations were commenced early in that year, the examination of sources and collection of samples being undertaken by the Engineering Department, and the bacteriological analyses being made at the Lawrence Experiment Station. In reporting to the legislature upon this work in January, 1902, the following statements were made: "The outlets along the seashore, by which flats or tidal waters which are sources of shellfish used extensively for food were liable to be polluted, were deemed to be of great importance, on account of the direct injury to health that might result from the use of contaminated food; and the work of the Board during the past year has been directed principally to an examination of the conditions about sewer outlets into the sea and tidal waters." "The analyses have consisted in a determination of the presence or absence of the colon bacillus, the characteristic organism of sewage. In collecting the samples from the various flats and waters in which they occur it has been the custom in most cases to collect with the sample of shellfish a sample of the water upon or near the flats, and analyses have been made both of the water and the shellfish."

At the time these investigations were commenced, little was known about the bacterial flora of shellfish, about the viability of B. coli, the typhoid bacillus, and other organisms in shellfish or in sea-water; in fact it was not definitely known whether the shellfish would take up B. coli, and it was necessary not only to devise satisfactory methods of analyses, but also to conduct experiments to determine how the

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results of analyses should be interpreted. The analytical methods now in use have been developed gradually as the investigations have continued, and while based largely upon results obtained at Lawrence, parts of the methods in use in other laboratories have been adopted or modified when such procedure seemed an improvement. The interpretation of results also, while based upon the Lawrence experiments, have included the many excellent researches conducted both in this country and abroad. In view of the growing importance of the subject and in response to frequent requests for information concerning the methods in vogue in Massachusetts, it is the purpose of the writer in the present article to describe in detail the present practice in the collection and analysis of shellfish samples, and the interpretation now placed upon the results of those methods, and to supplement these remarks by notes as to certain modifications which suggest themselves. As in some instances these notes may be taken as criticisms of the really excellent work of other investigators, all references except to Massachusetts work have been purposely omitted.[1]

As stated previously, complete bacteriological analyses are also made of the samples of sea-water from the same sources as the shellfish samples, the methods of sea-water analysis being practically identical with those for waters of other types. While the water analyses have been of much value, together with the shellfish examinations, in determining the amount of pollution of the various sources examined, it would be unwise to depend upon sea-water analyses alone, since the character of such samples in the majority of cases is influenced by tidal flow, wind, currents, etc. On the other hand, the analyses of the shellfish themselves as made at the present time show the amount of pollution at the time they are gathered and the condition in which they would be marketed and consumed.

COLLECTION OF SAMPLES.

Samples are collected in sterile springtop glass preserve jars holding one pint.[2] In the earlier work, when sampling at growing

[1] A complete review of the literature concerning shellfish pollution investigations is given in a report made to the legislature in 1902, published as *Massachusetts Senate Document*, No. 336, 1902.

[2] For oysters, the openings in jars may be so small that only the smaller oysters can be introduced. As only a small proportion of shellfish samples in Massachusetts have been oysters, the use of the glass jars has been continued, small oysters being selected when it is necessary to collect them. Metal boxes, such as lunch boxes, might be used when large oysters must be collected. The collapsible kind could

grounds, a sterile spoon was used to pick up clams and place them in jars. This seems to be an unnecessary refinement, and in recent years the collector has used his fingers, and so far as could be determined the error or chance of contamination by so doing has been negligible. If sterile handling is considered necessary, the individual clams might be picked up with crucible tongs which could be sterilized by the alcohol method before use, or could be kept in a bottle of some sterilizing agent when not in use. The sample from each location should consist of about 15 individual clams, as some may be lost in transportation or during the analysis.

TRANSPORTATION OF SAMPLES.

It is desirable that samples reach the laboratory as soon after collection as possible. In the earlier work in Massachusetts samples were packed in ice during transit.[1] Experiments upon the viability of B. coli and B. typhosus in clams and oysters, showed that if the weather was not too warm shipment without ice did not affect the analytical results, and in recent years icing has been omitted on shellfish samples which are to reach the laboratory within 24 hours after collection. Possibly two or three days might elapse without seriously affecting the results, but the shorter period is preferable.

INSPECTION OF SAMPLES.

When samples are received at the laboratory they should be inspected, to see if in good condition. If in good condition the shells will be closed tightly or will close tightly as soon as the individual clam or oyster is touched. Individuals with shells open, or which do not close quickly on being touched are either dead or nearly so, and should be rejected.

TECHNIC OF TESTING.

Ten clams or oysters from each location are tested for the presence of B. coli in the shellwater and in the alimentary canal.[2] The

be conveniently sterilized and if wrapped in paper could be easily carried around or stored until needed. Perhaps a waxed cardboard box would serve the purpose as well, in which case new sample boxes could be used each time. The advantage would be, less weight and the elimination of cleaning and resterilizing.

[1] The shipping case used for shellfish samples packed in ice is similar in construction to the case used for water samples described by the writer, *Technology Quarterly*, December, 1908, p. 516.

[2] Investigations of the relative occurrence of B. coli in different portions of a large number of polluted clams and oysters showed that the test organism could be isolated from the shellwater with the largest percentage of accuracy, and with slightly less accuracy from the intestines (*Report Mass. State*

required number of fermentation tubes should be marked distinctly by some such method as that described later under "Recording" before starting. The technic of testing is as follows: The individual is removed from the sample jar with sterile crucible tongs, and washed with a stream of sterile water.[1] This sterile water should preferably be as hot as can be conveniently handled.[2] The clam being laid upon the left hand and held firmly, the fingers gripping the hinge end, the point of a sterile oyster knife is inserted between the shells and the muscle cut.[3] With a little experience, this may be done without injuring the body of the clam or oyster. The upper shell being removed, a portion of the liquor in the lower shell is now transferred to a fermentation tube with a sterile pipette, or a portion of this shellwater may be carefully poured directly from the shell into the tube. The latter method is much simpler than the use of pipettes, but requires that the shell be so handled in the previous operation that

Board of Health, 1905, pp. 430 ff.). Since in many methods of cooking, the shellwater is discarded, and on the other hand the shellwater or broth as it is often called is used alone in many instances, it was believed that valuable information would result from separate tests of both shellwater and intestines. The object in testing ten individuals separately is to permit the results of tests to be expressed quantitatively, thus showing to a considerable extent the degree of pollution. Determinations of the numbers of bacteria in the shellwater have been made from time to time but the numbers so determined apparently did not have sufficient diagnostic value to repay the extra labor involved in making them. During the past few years, however, counts of the total and red colonies on litmus lactose agar after 18 hours at 40° C. have been made a part of the routine analyses of samples of water and sewage and the results so obtained have proved a valuable addition to the usual determination of total bacteria at room temperature and qualitative tests for B. coli. No opportunity has been afforded to apply this method to analyses of shellwater, altho some similar counts of sea-water have been made. It is believed, however, that differential counts of this character might prove extremely valuable after their significance had been worked out, and it is quite possible that such counts might entirely supplant the more tedious and less accurate qualitative tests for B. coli as now applied.

[1] A wash bottle for sterile water designed especially for use in shellfish analyses was described by the writer, *Technology Quarterly*, December, 1908, p. 519.

[2] If preferred, the shellfish may be dried with a towel or with a clean sheet of filter paper, dipped in alcohol, and burnt off as described under "Sterilization of Instruments," after the shell has been washed, and the same procedure may be followed when the body has been removed from the shell and washed preparatory to dissection. This seems to be an unnecessary refinement, however, if the samples are carefully handled.

[3] In attempting to open oysters, the beginner would better have a heavy leather or cloth pad to protect the hand from injury in case the knife should slip. This pad may be covered by a sheet of clean waterproof paper to prevent carrying contamination from one sample to another if desired. With a little experience and careful handling this refinement is not necessary, as the pad or hand should never come in contact with the portion of shellfish which is used for inoculating the tubes. The difficulty of opening oysters has led some operators to kill the oyster by immersing in boiling water or laying on a hot plate. Such a process is objectionable on theoretical grounds as the test organisms may be killed also. If care is taken to check the application of heat immediately the shells start to open it is possible the harm done may not be serious and such a process might be allowed in urgent cases. It cannot be recommended. Another method practiced in some laboratories is to bore through the shells with a sterile drill. There is no objection to this if a mash sample of body and shellwater is desired. The separate testing of shellwater and intestine is of course impossible by this process.

the lip over which the liquor is poured has not been contaminated. The body is now washed with sterile water,[1] then while held with the fingers of the left hand, an incision is made with a sterile scalpel and a portion of the intestine transferred with sterile forceps to another fermentation tube,[2] care being taken not to touch the parts where the incision is made with the fingers or to contaminate it in any way. This procedure is repeated until 10 individuals have been tested from each sample jar.[3]

CULTURE METHODS.

The tests for B. coli are made by the well known Lawrence method in exactly the same manner as are similar tests for B. coli in water. Preliminary fermentations in dextrose peptone solution are incubated 18 hours at 40° C. Tubes showing fermentation are plated out on litmus lactose agar, and incubated 18 to 24 hours at 40° C. If red colon-like colonies develop, two or more are fished from each litmus lactose agar plate to agar streaks. If colonies of the sewage streptococcus type are abundant on the plates, or if agar streak is of the streptococcus type, this should be noted also.[4] If agar streaks show characteristics of B. coli after 18 to 24 hours at 40° C., transfers are

[1] See footnote 2, p. 81.

[2] In some laboratories it is the practice to transfer the shellwater and body of the shellfish to a sterile petri dish, and after mashing them up together to inoculate a single fermentation tube with the mixture. Experiments at Lawrence have shown that it is quicker and neater to perform the operations as described above, and if it is necessary to reduce the number of tests to be carried out to inoculate both the shellwater and intestine from each individual into the same fermentation tube. Mixing the contents of the two fermentation tubes after incubation is also practiced in some places, but while this process yields somewhat more information as to the presence of fermenting organisms than does the combined method, experience has shown that the chances of isolating B. coli from such a mixture are considerably reduced, and this is the ultimate aim of the tests. When separate tests of shellwater and intestines can be made, the additional information obtained will amply compensate for the extra labor.

[3] In some instances when a large number of samples was collected on the same day, or when the number of individuals in each jar was not enough, five individuals or even less have been tested. This reduces the quantitative accuracy of the results, and impairs the judgment of the extent of pollution. If the number of tests which can be made is limited by insufficient apparatus or by pressure of other work, combined inoculation of the same tube with both shellwater and intestine is preferable to cutting down the number of individuals tested.

[4] It has been the experience at Lawrence that the streptococcus type is present more frequently in the shellwater of clams, etc., from polluted sources, and in polluted sea-water than in any other class of samples, and as it quite commonly overgrows the colon and other fermenting types, the isolation of such types is at times almost impossible. While B. coli is considered a better index of pollution than is the sewage streptococcus, and while no direct search is made for the latter, the presence of the streptococcus type undoubtedly does indicate pollution and when observed on the litmus lactose agar plates or upon the agar streak it should be recorded. The appearance of the streptococcus on the plate or agar streak is so different from any other types occurring under the conditions imposed by the methods, that it is readily recognized and its confirmation by microscopical examination is entirely unnecessary. See *Report Mass. State Board of Health*, 1901, p. 406.

made to tubes of Dunham's solution, nitrate-peptone solution, dextrose-peptone solution, and gelatin.

Dextrose-peptone and nitrate tubes are incubated 18 to 24 hours at 40° C. after which they are tested respectively for presence of gas or for nitrites. The tubes of Dunham's solution are tested for indol after three days at 40° C. The gelatin tubes are incubated at 20° C. for 14 days, being inspected for liquefaction on the fourth, seventh, tenth, and fourteenth day.

STERILIZATION OF INSTRUMENTS.

The simplest and easiest method of sterilizing the tongs, knives, forceps, etc., used in shellfish analysis is by dipping them into wood or denatured alcohol and then passing them through the flame, allowing the alcohol to burn off naturally. This will effectually destroy small particles of infected material without drawing the temper of the steel. It goes without saying that the instruments should be clean before sterilizing by this process, as any large particles of flesh, etc., would only be sterilized upon the outside. It is better to use instruments composed entirely of metal as the alcohol is apt to soak into the seats of wooden or bone handles, which become charred after being burnt off a few times, allowing the steel blades to become loose.

MEDIA.

The media employed correspond essentially with those recommended by the Committee on Standard Methods of Water Analysis of the American Public Health Association as modified in 1906 and 1907. The principal difference consists in the use of a solution of one per cent dextrose and one per cent peptone (Witte) in tap water in place of the usual Smith's solution, so called, in the fermentation tubes. This medium, as noted by the writer elsewhere,[1] yields satisfactory qualitative fermentation tests, but as the amount of gas formed is much smaller than in media containing beef broth, measurements of gas ratio if they are desired are not as conveniently made. Its chief advantage lies in the greater ease of preparation and in the facts that its composition is more uniform than that of media containing meat, that no adjustment of the reaction is necessary, and that no change of reaction occurs during sterilization.

[1] *Report Mass. State Board of Health*, 1901, p. 399.

Another variation is in the agar media used for streak cultures which contains only one per cent of agar instead of one and one-half per cent as originally recommended by the Standard Methods Committee.

RECORDING AND REPORTING.

When collecting samples of shellfish from the gathering grounds it is better to indicate the sampling stations by letters or numbers, and record the location marks in a notebook together with the station number where they may be available for future reference. The Massachusetts practice has usually been to indicate the stations from which shellfish samples were collected by numbers, and stations from which water samples were collected by letters, locating these stations upon a map or chart which is filed with the analyses and a copy of which is included with the report.[1] On arriving at the laboratory, the jars of samples and the accompanying certificates showing their source are numbered serially. The last one or two figures of the sample number or the location number are marked on the fermentation tubes with a wax pencil when inoculating from the shellfish. In addition, the tubes for each sample are numbered serially from zero to nine, and the tubes inoculated with shellwater and intestine are marked *a* and *b* respectively to enable a complete record to be made of any peculiar characteristics of each individual. These marks may be carried throughout the confirmatory tests if desired. At Lawrence, where an exact record is kept of the detailed behavior of every culture submitted to confirmatory B. coli tests, it has been found more convenient to have a series of culture numbers and to substitute these numbers for the sample marks when agar streak cultures are fished, the sample marks being entered in the B. coli culture book against the culture numbers.

In reporting, the results of shellfish analyses are expressed as percentages of the whole number of individuals tested from each sample, the expression of percentages being facilitated by the fact that ten individuals were tested in each case. The report shows:

I. Percentage of individuals from each sample which ferment dextrose.
 - *a*) shellwater.
 - *b*) intestine.
 - *ab*) both shellwater and intestine.

[1] Examples of the use of maps upon which the location of sampling stations are marked are to be found in the "Report on Pollution of Boston Harbor," *Report Mass. State Board of Health*, 1905, pp. 413–26.

II. Percentage of individuals from each sample in which B. coli was found present.
  a) shellwater.
  b) intestine
  ab) both shellwater and intestine.

III. Percentage of individuals from each sample in which sewage streptococcus was noted.
  a) shellwater.
  b) intestine.
  ab) both shellwater and intestine.

INTERPRETATION OF RESULTS.

Experiments at Lawrence and elsewhere have shown that the relative viability of B. typhosus and B. coli in clams and oysters and in sea-water, as well as under a variety of other conditions, is very similar and that the presence of B. coli may reasonably be considered to represent the possible presence of B. typhosus. Furthermore, the experiments have shown that these organisms may live in shellfish kept under market conditions for many days, and that the methods of cooking ordinarily employed frequently do not kill these organisms when they are present inside the shellfish.[1] In addition there have been a number of well authenticated epidemics of typhoid fever traced to the eating of polluted shellfish. On the other hand, the vital statistics of cities and towns along the seacoast, where shellfish are plentiful and are consumed in considerable quantities, do not show any marked increase in typhoid fever, other conditions being equal, over places in the interior where the consumption of shellfish is relatively small. And this is in spite of the fact that in some instances the shellfish consumed locally are taken from sources that are grievously polluted.

The investigations at Lawrence showed that B. coli and the sewage streptococcus are absent, or are present in only a small proportion of samples collected from sources which are remote from pollution. Sources which are absolutely free from all suspicion of chance pollution are rare, however, and the test organisms may be present in a small proportion of samples which would nevertheless ordinarily be considered entirely safe. On the other hand, the test organisms are always present in a majority of the samples from sources whose pollution is evident. Even the most seriously polluted sources, however, may yield a certain percentage of samples in which B. coli

[1] "Experiments on Relative Viability of B. coli and B. typhosus," *Report Mass. State Board of Health*, 1902, pp. 268–77. Experiments on "Length of Life of B. coli in Clams, Oysters, and Sea-Water and on Efficiency of Various Methods of Cooking Shellfish," *Report Mass. State Board of Health*, 1905, pp. 429–56.

are not present, or at least in which they cannot be demonstrated. For example, B. coli could not be found in a considerable percentage of samples of clams collected close to the outlet of a large city sewer, altho present in 80 to 90 per cent of samples from the same area at a distance of one-half to one and one-half miles from the sewer outlet.[1]

It should be stated in the strongest terms, that the determination of the presence or absence of B. coli in a single clam or oyster from a given source is of small diagnostic value if we may draw conclusions from the results of many thousand tests made at Lawrence during the last eight years. With a sufficient number of samples, the absence of B. coli, or of positive fermentations followed by an overgrowth of streptococcus, can safely be taken to indicate freedom from pollution, and when 10 individuals are tested, a negative test in 8 out of 10, or 80 per cent of the samples, can be assumed to be indicative of reasonable safety. Under the same conditions, a positive test in 50 per cent or more of the samples must indicate pollution of a more or less dangerous character. Between these extremes, and it is within this limit that a large proportion of sources will be found to fall, the interpretation is a question of individual judgment for which no hard and fast rules can be made. In such cases the sanitary inspection of the source must play an important part, and the extent and condition of the shellfish industry in that particular place, and whether the shellfish are to be eaten raw or are to be cooked before being consumed must also bear some weight if justice is to be accorded to both consumer and producer.[2]

[1] *Report Mass. State Board of Health*, 1902, pp. 260–62.

[2] Shellfish, such as clams, oysters, quahogs, scallops, and mussels, form an important part in the food supply, and the gathering of these shellfish is an industry in which many persons are employed. In Massachusetts, the value of the shellfish gathered along the shores amounts to about $500,000 per year or about one-tenth of the entire income from the fisheries, about one-half of this being from oysters, about one-fourth from clams and quahogs, and the remainder from scallops, mussels, etc. Altho the oyster catch is the most valuable, the great bulk of the food supply from this source is composed of clams and quahogs, of which some 400,000 bushels are gathered annually, while the oyster catch is only about 55,000 bushels, and the scallop catch about 90,000 gallons. About 90 per cent of the shellfish consumed in Massachusetts are produced within the state. Relatively a small proportion of the clams consumed are imported, while perhaps half of those gathered within the state are exported to other states. On the other hand, probably more than half the oysters consumed are brought in from outside, while only a small proportion of those gathered within the state are exported. For this reason the control of the pollution of the shellfish gathering grounds within the state is of the utmost importance as affecting the health of the community. As most of the gathering grounds are the property of the state or of the cities and towns, and as access to these is free to all, the investment required to engage in these industries is small while the returns on that investment are quite large. The industry is not organized and is composed largely of individuals who work separately or in small groups and who in the majority of cases market their catches themselves.

THE PRECIPITIN REACTION IN TUBERCULOSIS.*

A. E. Porter.

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In the serum diagnosis of tuberculosis we are met with difficulties caused either by the slight and varying degree of immunity present, or by complexity of method. On account of the want of constancy which these signs are apt to exhibit, it appears desirable that several should be at our disposal, and that these be as simple and as reliable as possible. At present only one method of serum diagnosis is made use of to any extent for the detection of tuberculosis, Wright's[1] opsonic test, and even its practical usefulness is still under discussion. Agglutination with so-called homogeneous cultures has given good results in the hands of its discoverer, Arloing,[2] but has not yet succeeded in being adopted more generally. Koch's[3] agglutination method has not been able to find its way any better into the clinical laboratories. Therefore Bonome's[4] initiative in trying to identify and to differentiate various strains of tubercle bacilli by means of the precipitin test met with general interest.

Trying to adapt Bonome's method to clinical exigencies, I have attempted to discover how far the precipitin test in tuberculosis is to be depended upon, and how its technic may be best simplified.

For this purpose I have tested 682 human sera, of which 381 were tuberculous, and 301 normal, at least normal in so far as they were taken from persons in whom tubercle was not suspected.

After many trial experiments in which I tried different tuberculous extracts, and different dilutions of serum, I finally adopted the following procedure:

I took 1 c.c. of Koch's bacillus emulsion and made it up to 20 c.c. with sterile water, shaking and keeping it at 37° for 24 hours. I then separated it into two halves to each of which I added water containing sufficient salt to render the whole isotonic, and in one case sufficient phenol to give a percentage of 0.5. Each part was in this

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[1] *Proc. Roy. Soc., Lond.*, 1904, 73, p. 357.

[2] *Compt. rend. de l'Acad. Sci.*, 1898, 126, p. 1398.

[3] *Deut. med. Wchnschr.*, 1901, 27, p. 829.

[4] *Centralbl. f. Bakt.*, Orig., 1907, 43, p. 391.

way made up to 25 c.c., diluting the original bacillus emulsion to 1:50, both being isotonic, but one having in addition 0.5 per cent phenol. After 12 more hours at 37°, each part was filtered through porcelain.

Altho I kept to this method throughout, I could not discover that bacillus emulsion added immediately to saline solution or to carbolized saline solution, or even where these solutions were added to ground bacteria, and kept at 37° for 36 hours and then filtered, did not give perfectly satisfactory results.

Every week fresh extracts were made.

The greatest difficulty lies in the cleaning of the tubes, which had each to be minutely examined before use. New tubes, I first brushed out with a mixture of soap and fine sand. After washing with water, dilute sulfuric, and distilled water, the tubes were boiled in distilled water. Immediately after use they were washed out with water, and cleaned with a metal rod around which cotton wool had been tightly wrapped, while the tubes were full of water. The narrowest tubes possible were used.

The serum was diluted by means of a capillary pipette, as narrow in bore as possible, from which one drop of serum was allowed to fall into a tube. Twenty drops of 0.5 per cent NaCl were added from the same pipette, and the contents of the tube mixt. Seven drops were taken from this dilution and placed in a second tube, and seven drops into a third. In this way each serum was diluted 1:21, and distributed equally between three tubes. If by chance rather less than one drop of serum was secured, the capillary tube was graduated by means of a mark and the serum diluted in this way, one part of serum to twenty of saline solution, mixt in the first tube, and after that taken up by the pipette and measured in drops if these would divide in three. When all the tubes had been supplied with serum in this way, an equal number of drops of (1) carbolized tuberculous extract, (2) uncarbolized tuberculous extract, and (3) of a solution containing only 0.5 per cent phenol and 0.85 per cent NaCl solution, was dropped into each of these three tubes. The final mixture therefore contained equal parts diluted 1:20 serum, and diluted 1:50 tuberculous extract, or carbolized saline solution.

The contents of the tubes were not allowed to be exposed except when the serum or extract was added.

The serum was rejected if it was not absolutely clear after dilution, or if it was hemolytic or even highly colored.

This dilution of 1:20 is not critical in any way. Other dilutions, if not too weak, act well enough. I chose this dilution because, while not too weak, it yet gave a convenient amount of fluid in the tube for a suspended precipitate to be easily examined.

Stoppered with sterile wads, the tubes were then transferred to the incubator, and left at 37° for 12 hours. I used at first four hours at 37° and 12 at room temperature, but found that this was insufficient. The precipitate after four hours has not in most cases developed, and room temperature is not sufficient to encourage it. On the other hand more than 12 hours is unnecessary. It appears that all sera do not possess an inherent tendency to precipitate. Sera which did not precipitate in 12 hours, did not do so in one or even two weeks.

On one occasion only was I able to obtain a precipitate almost instantaneously when using Fornet and Müller's[1] method of keeping the antigen and the antibody in two separate layers. The majority of

[1] *Ztschr. f. biol. Technik u. Methodik*, 1908, 1, p. 201.

my reactions were too slow in appearing, so that the advantage of this delicate method was lost. No doubt many fine precipitates were missed on this account. Fornet and Krencker[1] however have been able to use this method successfully in tuberculosis.

The serum, after 12 hours' incubation, may remain clear throughout, or it may appear clear but show a slight deposit at the bottom, or in addition to a deposit it may exhibit a more or less intense suspended precipitate.

On account of the extreme slightness of the deposit occasionally, I carried out the test, almost throughout, without knowing whether I was examining tuberculous or normal sera.

Because of the fact that different salts have been shown, especially by the classical experiments of Pauli,[2] greatly to influence proteid precipitations, I have separated the records of male and female sera, as their salt content, according to the textbook of Simon,[3] is very different.

NORMAL SERA.

The normal sera numbered 301, of which 174 were male, and 127 female. Fifty-three sera came from healthy individuals, 248 from persons suffering from other diseases, which included such diseases as carcinoma, diabetes, blood diseases, pneumonia, rheumatic fever, etc.

TABLE 1.
NORMAL SERA.

	No. of Noughts	Percentage of Noughts	No. of Deposits	Percentage of Deposits	No. of Suspended Precipitates			Percentage of Suspended Precipitates	Tested with
					+	++	+++		
Male sera	121	69.5	28	16	17	7	1	14.3	(1)
	121	69.5	28	16	17	7	1	14.3	(2)
	122	70.1	28	16	17	7	..	13.9	(3)
Female sera ..	85	66.9	30	23.6	11	1	..	9.4	(1)
	86	67.7	29	22.8	11	1	..	9.4	(2)
	90	70.8	27	21.2	9	1	..	7.9	(3)
Total.........	206	68.4	58	19.2	28	8	1	12.3	(1)
	207	68.7	57	18.9	28	8	1	12.3	(2)
	212	70.4	55	18.2	26	8	..	12.2	(3)

Each serum was tested with (1) a 0.5 per cent carbolized tuber-

[1] *Deut. Archiv f. klin. Med.*, 1909, 97, p. 282.

[2] *Archiv f. Physiol.*, 1899, 78, p. 315.

[3] *A Manual of Clinical Diagnosis*, Phila., 1904, p. 30.

culous extract in 0.85 per cent NaCl, (2) an uncarbolized tuberculous extract in 0.85 per cent NaCl, and (3) 0.5 per cent phenol in 0.85 per cent NaCl.

The highest percentage of precipitates was given by carbolized tubercle extract. In a few cases a precipitate, present in (1) and (2), was not present in (3), or in (1) and (3) and not in (2). In almost all cases, however, while the precipitate with carbolic alone

TABLE 2.
Tuberculous Sera.

	No. of Noughts	Percentage of Noughts	No. of Deposits	Percentage of Deposits	No. of Suspended Precipitates			Percentage of Suspended Precipitates	Tested with
					+	++	+++		
EARLY CASES (110)									
Male (55)	8	14.5	25	45.4	17	5	..	40	(1)
	9	16.3	24	43.6	17	5	..	40	(2)
	17	30.9	16	29	18	4	..	40	(3)
Female (55)	12	21.8	26	47.3	16	..	1	30.9	(1)
	12	21.8	26	47.3	16	..	1	30.9	(2)
	19	34.5	19	34.5	16	1	..	30.9	(3)
CHRONIC CASES (191)									
Male (103)	12	11.6	27	26.2	40	16	8	62.1	(1)
	15	14.5	25	24.2	39	16	8	61.1	(2)
	18	17.4	24	23.3	39	15	7	59.2	(3)
Female (88)	7	8	24	27.2	34	18	5	59.1	(1)
	8	9	28	31.8	29	18	5	59.1	(2)
	15	17	27	30.6	32	12	2	52.3	(3)
ADVANCED OR ACUTE CASES (80)									
Male (33)	11	33.3	17	51.5	3	..	2	15.1	(1)
	11	33.3	17	51.5	3	..	2	15.1	(2)
	18	54.5	10	30.3	3	..	2	15.1	(3)
Female (47)	9	19.1	25	53.2	11	1	1	27.6	(1)
	11	23.4	24	51	11	1	..	25.5	(2)
	17	36.1	19	40.4	10	1	..	23.4	(3)

was generally rather less intense, still a precipitate which appeared in the uncarbolized tubercle extract appeared also in the carbolic solution alone which contained no tubercle extract, the serum in all three cases being affected together.

The question of whether these precipitates are specific, caused by the union of precipitinogen and precipitin, is rather intricate. The recent discussion on the specificity of other precipitins shows how undecided this question still is. On the other hand, Naegeli[1] reports that 97 per cent of autopsies carried out by him have demonstrated

[1] Naegeli, quoted from the *Brit. Med. Jour.*, 1909, 2, p. 904.

scars of former tuberculous lesions. Dr. Philip,[1] also, calculates that one-third to one-half of all persons are tuberculous. Bonome[2] believes that human serum contains normally traces of tubercle precipitin.

I had almost every one of the precipitating normals examined for signs of the disease, but none were to be found. Some of the largest precipitates were given by apparently perfectly healthy persons.

Since Bonome, two interesting papers have been recently published on this subject, by Szaboky,[3] and Stoerk.[4] The cases of Szaboky precipitated the more the farther the disease was advanced. He obtained from seven advanced sera results which were never nil or even weak. This however has not been my experience. A greater percentage of my advanced cases gave no precipitate (26.2 per cent), than of my early cases (18.1 per cent).

The chronic cases, however, yielded remarkable results. Only 9.8 per cent were completely clear, and altogether the precipitates formed were unusually intense.

This was generally but not always the case where the patient was in rather good condition, or where he had suffered from the disease some years previously, and it was apparently arrested.

THE PRECIPITIN REACTION IN PERSONS TREATED WITH TUBERCULIN INJECTIONS.

Twenty-five cases, 20 of which were chronic, were being treated with injections of Beraneck's or Koch's tuberculin, at the time when the serum was taken. The number of cases is perhaps somewhat few to judge from, but the percentage of sera reacting did not show any noticeable increase, altho perhaps there was some increase in intensity.

TABLE 3.

SERA FROM TUBERCULIN TREATED CASES.

	No. of Noughts	Percentage of Noughts	No. of Deposits	Percentage of Deposits	No. of Suspended Precipitates			Percentage of Suspended Precipitates	Tested with
					—	— —	— — —		
Male	2	15.4	3	23	7	1	..	61.5	(1)
Female	3	23.3	3	23.3	6	..	..	50	(1)

[1] *Brit. Med. Jour.*, 1906, p. 472.

[2] *Centralbl. f. Bakt.*, 1907, 43, p. 391.

[3] *Ztschr. f. Tuberk.*, 1909, 14, p. 169.

[4] *Wien. klin. Wchnschr.*, 1909, 59, p. 417.

It is interesting that Wassermann and Bruck,[1] when using the complement-deviation test of Bordet and Gengou,[2] for the presence of tubercle antibody, found it only in cases treated with tuberculin. This observation is supported by the experiments of Citron[3] and Lüdke,[4] but Weil and Straus[5] and Czastka,[6] using the same method of complement-deviation, have been successful in demonstrating antibodies in the serum of cases of tubercle which had not been treated with tuberculin.

THE PRECIPITIN REACTION AND THE V. PIRQUET TEST.

Twenty-five cases had been vaccinated after v. Pirquet. Twenty-one of these gave a positive reaction, four were negative.

TABLE 4.

Noughts	Deposits	Suspended Precipitates			v. Pirquet Test
2	7	10	1	1	+ cases
..	3	1	..	..	− cases

One normal (?) person, in whom no tubercle could be detected (Dr. M.), was positive to v. Pirquet, and gave a slight deposit in answer to precipitinogen.

It is interesting that all the four cases which had been diagnosed clinically as suffering from tuberculosis, but which were negative to the v. Pirquet test, all gave a positive result to the precipitin test.

COMPARISON OF NORMAL AND TUBERCULOUS SERA.

If the results from all 381 tuberculous sera are put together and compared with the 301 normals, the difference is very striking. The

TABLE 5.

Comparison of Normal and Tuberculous Sera.

Kind of Serum		Percentage of Noughts	Percentage of Deposits	Percentage of Suspended Precipitates	Tested with
Male	Normal	69.5	16	14.3	(1)
	Tuberculous	16.3	36.1	47.6	(1)
Female	Normal	66.9	23.9	9.4	(1)
	Tuberculous	14.7	39.4	45.8	(1)
Total	Normal	68.4	19.2	12.3	(1)
	Tuberculous	15.4	37.8	46.7	(1)

[1] *Deut. med. Wchnschr.*, 1906, 32, p. 449.

[2] *Compt. rend. de l'Acad. Sci.*, 1903, 137, p. 351.

[3] *Berl. klin. Wchnschr.*, 1907, 44, p. 1139.

[4] *Münch. med. Wchnschr.*, 1908, 55, p. 783.

[5] *Wien. klin. Wchnschr.*, 1908, 52, p. 1059.

[6] *Ibid.*, 1908, 21, p. 877.

normal sera gave a negative result in 68.4 per cent of cases, whereas only 15.4 per cent of the tuberculous sera did not precipitate. The number of normal sera giving a suspended precipitate was 12.3, as against 46.7 per cent of the tuberculous sera.

It will be noticed that male and female sera do not differ to any extent.

THE PRECIPITIN TEST AND COMPLEMENT-DEVIATION.

One curious and rather embarrassing fact is apparent in the foregoing records, namely, that where even a considerable precipitate is given by uncarbolized extract, or carbolized extract, it is also given by carbolic and NaCl alone. This has also been noticed by Stoerk,[1] 60 per cent of whose tuberculous sera behaved in this way.

Moll,[2] and Welsh and Chapman[3] have attempted to prove that the antigen acts as a precipitant without taking any part in the precipitate, but remaining free in the upper fluid. Are we to suppose that the precipitin reaction is of so simple a nature that the precipitinogen can be replaced by such simple chemical agents as phenol in the case of tubercle? Does the presence of precipitin mean simply an inherent tendency toward precipitation? It became necessary to make some attempt to discover whether this precipitation was due to a true precipitin reaction or not.

According to Muir and Martin[4] and Neisser and Sachs,[5] complement-deviation is a much more delicate test for the presence of antibodies than is a precipitate reaction.

Complement is not deviated in a serum containing precipitin, or again in one containing precipitinogen. In the presence of both however in suitable proportions, complement is deviated. According to Moll, or Welsh and Chapman, this should be due to the precipitated precipitin; according to Bordet and Gengou, to the union of antigen and antibody.

I accordingly attempted to discover whether complement was deviated at all by these precipitates, and if so, whether the amount of deviation had any relationship to the amount of precipitate.

I took tubes containing mixtures of diluted serum and tubercle

[1] *Wien. klin. Wchnschr.*, 1909, 59, p. 417.

[2] *Beiträge Chem. Phys.*, 1903, 4, p. 578.

[3] *Proc. Roy. Soc., Lond.*, 1906, 78, p. 297.

[4] *Jour. of Hyg.*, 1906, 6, p. 1181.

[5] *Berl. klin. Wchnschr.*, 1905, 42, p. 1388.

extract, or carbolic solution, which had been already at 37° for 12 hours. Having noted down the amount of precipitate in each, I then added 0.15 c.c. of human complement, shaking to mix, and placed them again in the incubator at 37° for one hour. After this I added 0.25 c.c. of amboceptor and 1 c.c. of one per cent suspension of ox corpuscles and replaced them in the incubator for three hours with controls.

Deviation of complement did actually occur in certain cases, but had no relation to the amount of precipitate present.

All sera, which I had examined in this way, which had precipitated with the carbolic saline solution alone, did not deviate complement in the slightest.

On the other hand sera, and especially advanced case sera, in which I had not been able to detect the very faintest signs of a precipitate or deposit whatsoever, did deviate the complement completely, or almost so. Altogether 15 advanced case sera, eight of which had yielded no precipitate, were tested in this way and all deviated complement in the cases where tubercle extracts, carbolized or uncarbolized, had been used. Where carbolic saline alone had been added, however, precipitate or no precipitate, no deviation occurred.

From one point of view this observation may appear against the conception of Moll, and Welsh and Chapman, because the deviation in these unprecipitated advanced case sera was not due to a precipitated antibody. It was also not due to an unprecipitated antibody, because unprecipitated antibody was also present in the corresponding carbolic-alone test, where also no precipitate had occurred.

It appears possible that the precipitin reaction depends upon two processes: (1) the union of antigen and antibody, (2) the precipitation. In these advanced sera, for some reason, the second process may not have taken place.

Altho this explanation agrees best with the classical theory of the relationship between complement-deviation and the precipitin test, analogous and rather striking phenomena have been described by Uhlenhuth,[1] and Muir and Martin,[2] which appear perhaps to render another view possible. Uhlenhuth observed complement-deviation

[1] *Deut. med. Wchnschr.*, 1906, 32, p. 1244. [2] *Jour. of Hyg.*, 1906, 6, p. 1181.

in a serum immunized against the proteid of the lens of the eye, which is incapable of forming a precipitin antibody, or at least of precipitating with its antiserum. Muir and Martin were able to obtain a serum which had been immunized against another closely related species. This serum deviated complement when mixt with the antigen but did not precipitate. It appears at least possible that other bodies exist in an antiserum which can deviate complement. An actual precipitate is evidently not necessary.

However, complement was not deviated in the presence of the antiserum alone.

TABLE 6.

No. of Serum	Kind of Serum	Test with T. B. Extract	Test with Phenol	Hemolysis	Opsonic Count
1	tuber.	—	deposit	complete	0.9
2	"	—	—precipitate	"	0.3
3	"	—	"	"	0.45
4	"	—	deposit	"	0.8
5	"	—	—pre ipitate	"	0.66
6	"	—	"	"	0.43
7	"	—	"	"	0.46
8	"	—	—precipitate	"	0.17
9	"	—	"	"	0.07
10	"	—	"	"	0.06
11	"	—	"	"	0.1
12	"	—	"	"	0.33
13	"	—	"	"	0.2
14	"	—precipitate	—	trace	0.1
15	"	deposit	—	"	0.23
16	"	—	deposit	complete	0.46
17	normal	—	clear	"	1.17
18	"	deposit	—	partial	0.9
19	"	—	deposit	complete	1.16
20	"	—	"	"	1.1
NaCl					0.23
Control serum 3:17					1.07

Putting these facts together, Moll, and Welsh and Chapman find that when antigen and antibody are mixt, the resulting precipitate may contain no precipitinogen. My results go to show that a precipitate may occur when no antigen is present at all.

Muir and Martin, and Uhlenhuth have proved that deviation may occur without precipitation; my results, that in addition, precipitation may occur without deviation.

Muir and Martin,[1] and Heantjens[2] have showed that opsonin, like complement, is deviated by specific precipitates, formed by the union of antigen and antibody.

[1] *Brit. Med. Jour.*, 1906, 2, p. 1783.

[2] *Münch. med. Wchnschr.*, 1907, 54, p. 561.

I was anxious to discover whether opsonin behaved also like complement in relation to these non-specific precipitates between phenol and certain sera, that is to say, unaffected by them.

For this purpose I took away 0.05 c.c. of the mixture containing complement and opsonin which had been left in contact with the precipitate for one hour at 37°. The tubes were then supplied with immune serum and corpuscles and put into the incubator at 37° for three hours. Meanwhile an opsonic experiment, done with tubercle bacteria in the method of Wright, was carried out with the small portions of 3:17 diluted serum collected. The results shown in Table 6, p. 95, were obtained.

It appears that altho opsonin is perhaps better absorbed by a slight precipitate if antigen and antibody are both present, it may also be completely deviated by an intense non-specific precipitate, which leaves the complement unaffected.

This interesting fact, that opsonin was absorbed by the non-specific precipitate while complement was not absorbed, is in line with the other differences of behavior between these two bodies which have been pointed out by Fornet and myself.[1]

THE NON-SPECIFIC PRECIPITATE.

In describing the precipitate given between phenol and certain sera as non-specific, I do not wish to infer that the precipitate given with tubercle extract was necessarily specific.

The cause of this non-specific precipitate is difficult to explain. Erich Stoerk[2] states that a reaction may occur in cancer and diabetes. In neither cancer nor diabetes have I found the reaction. In pneumonia and rheumatic fever it was also wanting. He also believes that it may be due to a fatty diet. I have attempted to discover a relationship between diet and precipitation, and have utterly failed.

For example, 40 poor dispensary patients, living at home on a poor diet (24 of them early cases, who precipitate less regularly), gave no precipitate in 20 per cent of cases, which is rather above the average (15.4 per cent). They yielded however a higher percentage

[1] Fornet and Porter, *Centralbl. f. Bakt.*, Abt. I, Orig., 1908–9, 48, p. 461.

[2] *Wien. klin. Wchnschr.*, 1909, 59, p. 417.

of suspended precipitates more or less marked, 50 per cent in comparison with the average 46.7 per cent.

Again, 14 normal persons, living in a sanatorium and fed upon the same diet as the tuberculous patients, gave no precipitate in 11 cases, a faint deposit in 3 cases. Of 100 tuberculous patients in the same institution, 12 gave no precipitate, while 53 gave a more or less marked suspended precipitate.

I have noticed in several instances that tuberculous patients fed entirely upon milk gave a precipitate while non-tuberculous fed entirely upon milk gave none. The excess of milk which is often characteristic of a consumptive's diet, by altering the calcium content of his serum, cannot very well be credited with encouraging this reaction. Of 30 non-tuberculous persons fed entirely upon milk, 80 per cent were negative to the test, which is above the average for non-tuberculous persons, 68.4 per cent. I could in fact discover no relation between the precipitation and diet.

I have attempted to discover whether the addition of various simple salts, such as calcium, sodium, magnesium salts, chlorides, and sulphates, in a concentration up to $n/4$, could in any way induce a precipitate, which could not be called forth by tuberculous extract or carbolic alone. The only effect which I was able to notice was the natural one of a tendency to keep the precipitate slightly better in suspension, as might have been expected from the slight increase in density of the solution. In no case did a serum, which gave a negative result with tubercle extract or carbolic alone, precipitate when these salts were added, even after a week at 37°, nor did these salts hinder the precipitation as far as I could find. The reason for this non-specific precipitation requires further study.

Whatever the cause however, the fact that so large a percentage of tuberculous sera yield it on the addition of carbolic, is quite sufficient to warrant the use of such a simple and convenient test. The serum requires only to be diluted to say 1:20 by means of a capillary pipette, and added, equal parts, to a 0.5 per cent solution of phenol in 0.85 per cent NaCl solution.

In order to make certain that the serum contains the true antibody, a complement-deviation test, such as that of Wassermann and Bruck, seems advisable. The use of the tubercle extract in a precipitate

test is hardly necessary when the simple and convenient carbolic NaCl solution alone can act almost as well.

I wish to express my thanks to Dr. Macgowan, for immune serum, also to the following physicians for their courtesy in giving me all facilities: Drs. Claude Ker, Guy, Wilson, Selkirk, Maclaren, Krause, Langwill, Boyd, and Ballingall-Watson; also to Dr. Fornet and Dr. Cramer, for their encouragement and advice.

INJECTIONS OF HOMOLOGOUS STREPTOCOCCI KILLED BY GALACTOSE IN THE TREATMENT OF SUPPURATIVE COMPLICATIONS OF CONTAGIOUS DISEASES.*

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EVER since the treatment of infections by the injection of dead bacteria was first urged by Wright, interest in this method has been great, but the early reports of brilliant results have not met uniform confirmation. There is on record, however, a sufficiently large number of cases in which good results have followed the use of bacterial inoculations to justify further study of this subject, altho the number of cases in which the most careful use of these methods has failed to produce good results suggests the possibility that in a certain number of instances the good results obtained were merely coincident. Under such circumstances, the careful study of a few cases would seem to be of more value than the routine treatment of a larger number, since there are a great many factors in each case, an inadequate appreciation of which might easily lead to unjustifiable conclusions.

Levy, Blumenthal, and Marxer[1] killed tubercle, typhoid, and glanders bacilli with chemically indifferent agents, such as sugars, glycerin, and urea, and found that considerable quantities of such dead culture could be injected into animals without unpleasant results, and that the injections were followed by marked immunity for the corresponding living bacteria. Weaver and Tunnicliff,[2] in this laboratory, studied the difference in the degree of immunization against living streptococci induced in rabbits by injections of streptococci killed by heat, and by suspension in 25 per cent solution of galactose at 35° C. They tested the immunity by injecting living cultures and by determining the opsonic index. They found that whereas the injection of heat-killed streptococci produced little if any increase in the opsonin, and failed to protect the animals against

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[1] *Centralbl. f. Bakt.*, Abt. I, Orig., 1906, 42, p. 265.

[2] *Jour. Infect. Dis.*, 1908, 5, p. 589.

subsequent injections of living streptococci, or even made them more susceptible, similar injections of galactose-killed streptococci produced a substantial increase in the amount of opsonin, and rendered the animals more resistant than the controls to subsequent injections of homologous living streptococci. They also report two cases of chronic streptococcus infection, one of post-scarlatinal mastoiditis and otitis media and one of chronic erysipelas with acute exacerbations, both of which recovered while under treatment with galactose-killed homologous streptococci.

In a former series of cases Dr. Weaver and I studied the effect of injections of heterologous galactose-killed streptococci in scarlatina and in erysipelas, with reference to their power (*a*) to prevent suppurative complications or to ameliorate the course of the disease when injected during the acute stage of the disease, and (*b*) to modify the course of suppurative complications when administered later. Injections were given in 128 cases, and the following conclusions were reached:

1. The injection of polyvalent, heterologous streptococci, killed by chemically indifferent agents, during the acute stage of erysipelas, has no appreciable effect on the course of the disease. In cases running a prolonged course, such injections appear to exert a favorable influence.

2. The injection of such streptococci during the early stages of scarlet fever does not prevent the later development of local streptococcus complications, altho they may appear a little later in the disease.

3. The injection of such killed streptococci in scarlatina, after local streptococcus complications have developed, exerts considerable influence in hastening recovery. The later the complications appear, and the more chronic the complications are at the time of the injections, the better are the results following the injections. Of nine cases injected during the first week of the fever, only one showed prompt improvement, while of 23 cases injected later, 10 (44 per cent) showed prompt improvement.

4. Homologous streptococci are probably preferable for preparing the material for injection both in protracted, chronic, and recurrent cases of erysipelas, and in cases of scarlatina with local streptococcus complications.[1]

In order to study this matter further, careful observations were made on a new series of cases, comprising two cases of erysipelas, one recurrent, and one recurrent and relapsing, and 12 cases of scarlatina, of which five were complicated by diphtheria, two by measles, and one by measles, diphtheria, and chicken-pox. The cases were studied in the contagious ward of the Cook County Hos-

[1] Weaver and Boughton, *Jour. Infect. Dis.*, 1908, 5, p. 608.

pital, and grateful acknowledgment is hereby made to Dr. Weaver, Dr. Baum, and Dr. Cameron for the opportunity. Swabs were taken from the discharges in each case, and plated on blood agar. Only those cases were used which showed a preponderance of streptococci in the discharge. Subcultures were made on blood-agar slants by inoculating from several typical hemolytic colonies on each plate, grown in the incubator for 24 hours, and washed off with 25 per cent solution of galactose, in which they were suspended for 48 to 72 hours in the incubator (until subcultures proved sterile), separated by centrifugation, and dried in vacuo over calcium chloride at room temperature. Appropriate doses were then suspended in normal salt solution, and injected hypodermically. Only those cases were selected which, judging from our previous experience, were "favorable," that is, cases in which a suppurative complication had become chronic, and the acute stage of the disease had passed, or cases that were distinctly septic. The probable reason that acute infections so seldom show a favorable reaction to bacterial inoculations is that the body is already overtaxed in the production of antibodies, but in my experience "septic" cases seem to constitute an exception to this rule.

Weaver and Tunnicliff found that the maximum increase in opsonin produced in rabbits by an injection of galactose-killed streptococci is obtained on about the third or fourth day. Clinical observations accord closely with this view, altho I have occasionally observed cases in which improvement was noted as early as the next day after an injection. Furthermore, it is generally held that a second dose of dead bacteria rarely produces good results if given before the effects of the first have worn off, which usually means from 5 to 10 days, but cases have been reported in which a small daily dose over a period of one to two weeks is declared to be the cause of an eventual recovery.

In this series only those cases are considered as benefited which showed a sudden, marked improvement within four or five days after an inoculation. In a few cases in which two or more injections were given, it was particularly hard to judge the effect of treatment, as almost any rational method of treatment would surely show some improvement in two weeks' time. It was also noticed that in the

cases of scarlatina there was a marked tendency for suppurative complications to disappear about the sixth week of the disease, whether treated with inoculations or not. There is still another factor tending to confuse results. The cases reported in this series represent only about one-third of the cases for which material was prepared, the other two-thirds during the interval of 6 to 10 days required to prepare the streptococci by this method having either recovered completely, or improved to such an extent that it was not thought advisable to inject them. If this entire series of cases (all of them well marked cases) had been inoculated with "stock vaccines" when first seen, even if the doses had not produced any effect whatever, there would have been at least 67 per cent of good results that might have been attributed to the inoculations; adding the good results obtained in the 14 cases in this series the value of inoculation would be set much too high when the real facts are considered. The cases in this series are, then, a selected group which by comparison were refractory. Because of the various and confusing factors, great care must be taken in observing the cases if the results obtained are to be interpreted correctly.

The opsonic index was not determined systematically in all of the cases, because, after all, the clinical condition is the best criterion, opsonin being only one of the many antibodies produced in the course of an infection. The opsonic index, especially in long-standing suppurations, will often show considerable fluctuations which seem independent of the clinical condition. Moreover, it is questionable if the opsonic index obtained by using a laboratory strain of a bacterium is any index of the patient's resistance to a strain of the same bacterium which is virulent for him. Many observers have noted that the index is often higher for a virulent strain than for an avirulent strain of the same bacterium. This was observed in Case 4 of this series, the only one in which it was looked for.

The doses used in this series ranged usually from 50,000,000 to 500,000,000 cocci (tho once a dose of 10,000,000 was given, but without apparent effect). It is impossible to judge in such a small series of cases as to what the ideal dose would be, especially as there seem to be great individual variations. There seems to be no question that large doses may do harm. I have never observed any

harmful results to come from a single dose of 500,000,000 cocci, tho when such a dose was repeated in a week or so, I have sometimes thought that bad results occurred. I have obtained apparent good results three times with as small a dose as 50,000,000 cocci, but in none of the cases were the results permanent until subsequent larger doses had been given. With a single or an initial dose of 100,000,000 cocci the effects were more lasting. I have obtained good results with a single dose of 500,000,000 cocci, but such a large dose should not be repeated in less than two weeks, and possibly does no more good than a smaller dose. When an initial dose has produced good results, a second dose has not been observed to produce good results unless it is considerably larger than the first (three or four times as large), tho if the first dose has had no effect, a smaller increase will often be efficacious. Probably about 100,000,000 cocci (galactose-killed) is the best routine dose, for this dose is not large enough to produce any bad results, and yet is so small that it may be followed by a large dose if necessary without the risk of bad results. In two cases (3 and 4) it was observed that the first two doses produced good results, but that the subsequent doses, no matter what the size or the interval, were without apparent effect.

Case 1.—Girl, six years old, admitted September 6, 1908, sick three days. Scarlatina.

September 15. Both ears discharging freely.

September 28. Suppurating cervical gland opened.

October 26. Right mastoid opened, small amount of pus evacuated, and drainage established.

November 10. Ear and wound discharging freely. Opsonic index 1.4. Injected 500,000,000 own streptococci, galactose-killed.

November 11. Discharge increased, moderate local reaction. Opsonic index 3.9.

November 12. Discharge unchanged. Opsonic index 4.8.

November 13. Discharge less. Opsonic index 3.5.

November 14. Opsonic index 1.8. Injected 500,000,000 own streptococci.

November 15. Very slight local reaction.

November 16. Discharge remains moderate. Opsonic index 0.4.

November 17. " unchanged. Opsonic index 1.5.

November 18. " " " " 0.9.

November 19. " decreased. " " 4.9.

November 20. " increased. " " 1.0.

November 21. " unchanged. " " 0.8.

November 22. Symptoms developing in other mastoid; taken from hospital.

RESULT.—Probably the prolonged negative phase following the second large

injection made matters worse, altho the first injection seems to have produced good results.

Case 2.—Woman, 23 years old, admitted November 29, 1908, sick nine days. Scarlatina and diphtheria.

December 3. Right ear discharging.

December 22. Discharge profuse. Opsonic index 1.07. Injected 100,000,000 own streptococci.

December 23. Opsonic index 0.84.

December 24. Discharge stopped. Injected 100,000,000 own streptococci.

December 26. Opsonic index 1.8.

December 30. " " 1.82.

January 5, 1909. " " 2.66.

Result.—The inoculation apparently produced prompt and marked improvement. The opsonic index remained high so long as the patient was under observation.

Case 3.—Boy, 17 years old, admitted December 1, 1908. Diagnosis: Recurrent erysipelas with chronic nasal discharge and chronically enlarged and indurated upper lip.

First attack of erysipelas occurred in December 1907, lasted two weeks, and was of the usual facial type. When the erysipelas subsided, the upper lip remained swollen, and has remained so ever since. He had several attacks of erysipelas, but does not know how many, between December 1907 and July 1908, and each time the lip got worse during the attack, but subsided somewhat afterward, tho it never regained normal size. From July to December 1908 he was free from erysipelas, but the lip remained swollen and the nose remained sore and continued discharging. Another attack of erysipelas occurred December 1, 1908, which subsided in about one week. When first seen, December 18, the lip was swollen to about twice the normal size, a thin muco-sanguino-purulent nasal discharge was present, and the patient complained of constant soreness in the nose. Cultures made from the nasal discharge gave a practically pure growth of hemolytic streptococci. The patient received two injections of 100,000,000 streptococci each (December 29, 1908, and January 4, 1909), and nine days after the last injection he left the hospital.

The patient stated that he felt very much better for a few days after each injection, and two days after the first injection the soreness left his nose, and did not return. The amount of nasal discharge became less after the first injection, but was increased again a few days after the second. The lip became noticeably smaller about four days after the first injection, and continued to decrease in size while under observation. A very marked local reaction followed each injection, and what was apparently a sterile abscess formed at the site of the injection. Altho a complete recovery was not effected, the inoculation apparently exerted a very favorable influence, producing a marked improvement both in the local and in the general condition. The opsonic index determined daily during this period rose after the first injection from 0.84 to 2.75.

After the second injection the index rose to 4.7, but soon fell to about normal. On one occasion (January 5, 1909) it was observed that the opsonic index when determined with the patient's own leukocytes (1.21) was lower than when determined with normal leukocytes (2.04). One month later, when he was much improved, both serum and leukocytes were normal. This same phenomenon was observed in the next case and was now studied more carefully, the observations being reported in detail elsewhere. The leukocyte count varied between 8,800 and 14,750, averaging about 12,000, the

fluctuations following to a certain extent the fluctuations of the opsonic index, tho the correspondence was not close.

One week after leaving the hospital (January 21, 1909) he returned with a mild recurrence of the erysipelas. And it must be noted that in the five cases of recurrent erysipelas, inoculation of homologous or heterologous streptococci, tho often producing good results, never absolutely prevented recurrences. As soon as the acute symptoms had subsided, bacterial inoculations were resumed tho they seemed not to influence the local condition. During the month that he was in the hospital during and after this attack, he received five injections (increasing from 10,000,000 to 200,000,000), and three days after receiving the last one, he left the hospital. Every injection produced a severe local reaction, but the larger doses produced no more reaction than the smaller ones.

RESULT.—The first two injections apparently produced marked and permanent improvement. During his second stay in the hospital, he did not react nearly so well to injections, and altho he showed some improvement, it is doubtful if this can properly be ascribed to the inoculation.

Case 4.—Man, 32 years old, admitted December 10, 1908. Diagnosis: Recurrent relapsing erysipelas, otitis media. This case is reported in detail elsewhere, especial study having been made of the peculiar reactions which the patient's leukocytes showed in his own and in foreign sera in regard to phagocytosis. His first attack of erysipelas occurred in April 1908, the second in October 1908. Soon after this the left ear began to discharge, and continued to discharge until a couple of weeks before his death. The third attack occurred in December 1908, when he first came under observation. Subsequently he had three recurrences and four relapses. The attacks were all facial, and all except the first migrated down the back. The patient remained in the hospital until his death (May 20, 1909). He received six injections increasing from 50,000,000 to 500,000,000 cocci. The first two injections seemed to produce a favorable influence on both the local and the general condition, but the subsequent injections apparently produced no effect whatever. The injections did not prevent recurrences. The opsonic index following the first two injections rose from 0.36 to 2.5, but soon fell to about normal, and subsequently was not much influenced by the inoculations. Later in the disease the index remained constantly low (0.095 to 0.6). It was repeatedly observed that the index was lower when determined with the patient's leukocytes than when normal leukocytes were used. This was evidently dependent on some reaction between the patient's own leukocytes and serum, for the patient's leukocytes were practically normal when in normal serum, but much less active when in his own serum. It was observed, further, that the addition of a small amount of normal serum (human or horse) would greatly increase the activity of the patient's leukocytes when in his own serum, but when 10 c.c. of fresh normal horse serum was injected, a recurrence of the erysipelas was provoked. It was observed also that the index was higher for the homologous organism, grown under conditions calculated to preserve its virulence, than for a laboratory strain of the same organism, and while the addition of a small amount of normal serum would raise the index for the laboratory strain, it had the opposite effect for the virulent strain. The leukocyte count showed great variations which could not be related to the inoculation, to the opsonic index, or to the clinical course of the disease.

RESULT.—In this case the inoculation was apparently without marked or lasting effect; it cannot be said, however, that it produced any bad effects.

Case 5.—Girl, eight years old, admitted December 22, 1908. Scarlatina and diphtheria.

January 8, 1909. Ears discharging. Nephritis.

January 15. Measles.

February 12. Both ears and nose discharging freely. Opsonic index 0.72. Injected 100,000,000 own streptococci.

February 13. Slight local reaction. Opsonic index 1.1.

February 15. Discharge much diminished. General condition much improved, local reaction nearly subsided. Opsonic index 1.02.

February 16. Discharge slight. Opsonic index 0.8.

February 18. Opsonic index 0.67. Injected 100,000,000 own streptococci.

February 19. Opsonic index 1.26.

February 22. Discharge present but slight.

February 24. Chicken-pox. Injected 200,000,000 own streptococci.

February 26. Discharge much increased.

March 2. Discharge slight.

March 6. Moderate amount of bloody discharge. Injected 400,000,000 own streptococci.

March 8. Discharge much reduced. Great improvement in general condition.

March 10. Discharge remains about the same. Opsonic index 0.84.

March 11. Taken home.

Result.—In this case three of the four injections given apparently produced good results, but the intercurrent infection of chicken-pox interfered markedly with the course of the disease, and with the interpretation of the results.

Case 6.—Boy, six years old, admitted January 15, 1909. Scarlatina.

February 26. Measles.

March 10. Ear discharging freely.

March 30. Injected 500,000,000 own streptococci.

April 8. Discharge still present.

April 10. Discharge very slight—taken home.

Result.—The improvement in this case, occurring 11 days after the inoculation, cannot be attributed with any certainty to its effect.

Case 7.—Boy, six years old, admitted March 4, 1909. Scarlatina and diphtheria.

March 15. Ear discharging.

March 30. Ear discharge unchanged; injected 500,000,000 own streptococci.

April 1. Moderate local reaction. Discharge the same.

April 8. Discharge slight.

April 12. Discharge stopped.

Result.—The improvement in this case occurred so late as not to be fairly attributable to the inoculation.

Case 8.—Girl, eight years old, admitted February 23, 1909. Scarlatina.

March 1. Ear discharging.

March 30. Discharge continues. Injected 500,000,000 own streptococci.

April 1. Moderate local reaction, discharge slightly increased.

April 2. Discharge diminished.

April 3. Discharge stopped.

Result.—Inoculation apparently produced prompt and marked improvement.

Case 9.—Girl, five years old, admitted March 15, 1909. Scarlatina and diphtheria.

March 20. Nose and ears discharging freely.

March 30. Discharge continues; "septic"—seems moribund. Injected 100,000,000 own streptococci.

March 31. Died.

RESULT.—This case is included in the series for the sake of completeness. It cannot be said that any effect whatever was produced in this case by the inoculation.

Case 10.—Boy, 15 months old, admitted March 17, 1909. Scarlatina and diphtheria.

March 21. Aural discharge, soon becoming profuse.

April 24. Ears and nose discharging profusely. General condition very poor—semi-stuporous. Injected 50,000,000 own streptococci.

April 28. Marked improvement in general condition.

May 4. General condition again poor—looks moribund. Injected 50,000,000 own streptococci.

May 5. Condition somewhat better—removed from hospital. Further details of illness not available except that child recovered.

RESULT.—Inoculation apparently produced prompt and marked improvement in general condition.

Case 11.—Boy, three years old, admitted March 18, 1909. Diphtheria.

March 23. Scarlatina, moderate severity.

April 2. Both ears discharging.

May 4. Measles, moderate severity.

May 21. Discharge still continues. Injected 100 000,000 own streptococci.

May 26. Taken from hospital, discharge still profuse.

RESULT.—Inoculation apparently produced no result.

Case 12.—Girl, three years old, admitted May 15, 1909. Scarlatina.

May 29. Diphtheria.

May 31. Ear discharging.

June 6. Cervical abscess opened.

June 20. Ear discharge ceased, neck still discharging. Injected 100,000,000 own streptococci.

July 2. Neck discharge ceased, another small abscess on neck opened, which ceased discharging in a few days.

RESULT.—Inoculation apparently produced no effect.

Case 13.—Boy, six years old, admitted July 8, 1909. Convalescent scarlatina (fifth week ?), suppurative otitis media, subacute mastoiditis, suppurative cervical adenitis.

July 27. Profuse discharge from both ears and from wound in neck. General condition poor. Injected 50,000,000 own streptococci.

July 29. Marked improvement in local and general condition. From this time until date of dismissal the general condition continued to improve. The purulent discharge still continued tho much less in amount.

August 1. Cervical abscess healed.

August 4. Injected 100,000,000 own streptococci.

August 9. No appreciable result from last injection. Injected 200,000,000 own streptococci.

August 12. Aural discharge much diminished.

August 14. Aural discharge ceased.

Result.—Injections apparently produced prompt and marked improvement.

Case 14.—Boy, eight years old, admitted July 12, 1909. Diagnosis: Scarlatina, epilepsy.

July 15. Profuse discharge from nose and from both ears.

July 27. Discharge still profuse. Injected 50,000,000 own streptococci.

July 30. General condition much improved. Local condition unchanged.

August 4. Condition same. Injected 100,000,000 own streptococci.

August 9. Discharge somewhat less. Injected 250,000,000 own streptococci.

August 10. Discharge much improved.

August 14. Discharge ceased.

Result.—Injections apparently produced prompt and marked improvement.

Of these 14 cases, eight recovered completely while under observation, but in only five was the recovery due, apparently, to the inoculation. In the other three, the aural discharge diminished gradually, and finally stopped completely about 12 days after the inoculation, but in the absence of any sudden and well marked improvement, it is better to consider them conservatively. Of the six cases which did not recover completely while under observation, only two failed to show improvement and one of these died the day after the inoculation, so that perhaps it is not fair to place any stress on this one. Of the entire 14 cases, nine showed improvement following the injections, and five did not show improvement which could be ascribed to the injection; but of the five, three recovered under observation, one died the day after the injection, and one was taken from the hospital a few days after the injection, while the discharge was still profuse; of the nine which showed improvement, one (Case 1) later became worse, apparently because of two large injections at a short interval; one (Case 4) died four months later, the course of the disease during the latter part of the sickness being apparently uninfluenced, favorably or unfavorably, by the inoculation; one (Case 3) showed marked improvement after the first two injections and later, altho he continued to improve, the improvement was very slow and not plainly due to the inoculation; and in one (Case 12), altho the improvement was quite marked early, the course was much disturbed by an intercurrent infection of chicken-pox, which, in several cases in our experience, has invariably had the effect of making an aural discharge much more profuse and more resistant to treatment.

In the entire series of 14 cases, then, which may be considered as

more refractory than the average of cases of this class, we may fairly claim, as due directly to the inoculation, 36 per cent cured, 28 per cent improved, while 36 per cent were uninfluenced by the treatment. No ready explanation is at hand why some of the cases should respond to early treatment, but not to later treatment, altho they may go on to recovery, but apparently not influenced by the injections. On the other hand, some cases will not respond to the first or second injection, but will respond to later injections. In view of the generally accepted fact that acute infections, especially suppurations, do not usually respond well to bacterial inoculations, the behavior of cases of sepsis is without ready explanation, unless they be ascribed to error or coincidence, which seems not to be reasonable. In seven cases (three in this series, and four reported previously) in which injections were given in the presence of marked evidence of sepsis, prompt improvement (in one to three days) was noted in all but one, and that one was moribund when injected; another case that seemed moribund when injected showed a marked improvement the next day. In other cases in which the condition was not so marked, the patients would often declare that they felt much better after an injection, and the improvement was certainly not altogether psychic. This improvement in general condition was often noted to precede improvement in the local suppurative process by a day or two, and was sometimes present even when no change in the local condition could be detected. From purely anatomical considerations it is easy to see why a long-standing purulent discharge, especially one involving a bony cavity, as a mastoiditis or an otitis media, could not be expected to stop at once, no matter how much the bodily resistance might be raised.

CONCLUSIONS.

The injection of homologous galactose-killed streptococci in local streptococcus complications of scarlatina and of erysipelas in many cases appears to have a marked effect in hastening recovery. This series of 14 rather refractory cases shows 36 per cent of recoveries, and 28 per cent of improvements, due, apparently, directly to the inoculations. The average initial routine dose should be about 100,000,000 cocci. Large doses, especially if repeated at short intervals, are capable of producing harmful results.

The injection of small doses (50,000,000) of galactose-killed homologous streptococci appears to exert a favorable influence on septic conditions complicating scarlatina.

Some cases of streptococcus infection may show favorable results following a few early injections, when later injections appear to produce no reaction whatever.

INTERACTION OF SERUM LEUKOCYTES, AND BACTERIA IN PHAGOCYTOSIS AS OBSERVED IN A CASE OF RECURRENT AND RELAPSING ERYSIPELAS.*†

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Wright and his followers have maintained that the serum is the only variable element in phagocytosis *in vitro*, and that the source of the leukocytes is immaterial. Other investigators in the last three years have shown that this position is probably not tenable, and that there are variations in the phagocytic power both of the normal leukocytes and of leukocytes from cases of disease.

Rosenow[1] found that the leukocytes in pneumonia are perceptibly more actively phagocytic than normal leukocytes, a property which seems independent of the serum. They are more resistant to heat, and when heated to the thermal death point, and resuspended in serum, they are more actively chemotactic for pneumococci. Potter and Krumwiede[2] found that "just after the crisis or lysis the phagocytic power of pneumonic leukocytes increases rapidly, surpasses, and then later equals or becomes less than that of normal leukocytes."

Briscoe[3] states that the different varieties of leukocytes according to Arneth's[4] classification have distinctly different phagocytic powers, those of class III being most active, and those of class I least active. Pottenger[5] arrives at a similar conclusion from studying the leukocytes of normal persons and of cases of tuberculosis, and endeavors to explain the low resistance in certain cases of high leukocytosis by the fact that the new leukocytes may consist largely of classes I and II which have a much lower phagocytic power than the other classes so that the total phagocytic power of the blood may be much less than would seem to be indicated by the white count alone. Glynn and Cox[6] in a recent article have made careful and extended observations on the phagocytic power of leukocytes from three normal persons, from one case of mild chronic staphylococcus infection, and from a number of miscellaneous cases, infectious and otherwise. They find that the "inherent phagocytic power" of leukocytes (tested with Staph. albus and tubercle bacillus) varies fully as much as the opsonic index and probably more. In normal persons they found this "cytophagic index" to vary from 0.54 to 1.22 as compared with other normal persons.

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[1] *Jour. Infect. Dis.*, 1906, 3, p. 683.

[2] *Ibid.*, 1907, 4, p. 601.

[3] *Brit. Med. Jour.*, 1907, 2, p. 1493.

[4] *Die Neutrophilin weissen Blutkörperchen*, Jena, 1904.

[5] *Jour. Amer. Med. Assoc.*, 1909, 52, p. 1980.

[6] *Jour. Path. and Bact.*, 1909, 14, p. 90.

Ledingham[1] seems to have been the first one to point out a specific reaction between patient's leukocytes and patient's serum whereby a patient's leukocytes may be much more or less actively phagocytic in his own serum than are normal leukocytes under similar conditions, while in normal serum this difference may not appear. As a result of this relation the opsonic index will be higher or lower when determined with the patient's leukocytes than when normal leukocytes are used. He found in certain cases of myelogenous leukemia that the serum may have quite a normal opsonic content when tested with normal leukocytes, but a markedly subnormal one if tested with the patient's own leukocytes. On the other hand, in a case of chronic lymphatic leukemia, the polymorphonuclear leukocytes were much more actively phagocytic than normal leukocytes. It was also found that the leukocytes of the leukocytosis induced by cinnamate of sodium had much less phagocytic activity than normal leukocytes. Shattock and Dudgeon[2] found that lekocytes in infectious diseases may show considerably more or less phagocytic activity toward melanin and colon bacillus than normal leukocytes, and this relation may hold true when suspended either in their own serum or in a foreign serum (normal or from a case of infectious disease). They conclude that "immune or active cells, as compared with normal cells, altho they usually do more work in both immune and normal serum, do more work in the immune than in the normal serum." They consider this to be a peculiarity inherent in the cell itself, and not due to any stimulation by the serum. Bayly[3] tested the opsonic index (bacterium not mentioned) in 44 parturient women, and found that altho the index when determined with normal leukocytes was within normal limits in nearly all cases, yet when determined with the patient's own leukocytes, the index was lower in 38 cases; in four cases it was higher. He did not determine whether this was due to a decreased activity of the patient's leukocytes (as compared with normal leukocytes) when in their own serum, or to an increased activity when in a foreign serum. Parvu[4] studied phagocytosis of typhoid bacillus and staphylococcus in a case of myelogenous leukemia. His observations correspond with those of Ledingham, namely, that the opsonic index may be lower when determined with the patient's own leukocytes than when normal leukocytes are used. He also observed that many polymorphonuclear leukocytes lose the power of phagocytosis, and that this function is taken over to a considerable extent by the myelocytes. In his calculations he considered only the polymorphonuclear leukocytes as phagocytes. LePlay[5] studied the opsonic index in two cases of gonococcus infection, one of them complicated with typhoid fever. He found the index determined with the patient's leukocytes often lower than when determined with normal leukocytes. He says: "The index determined with the leukocytes of the patient and the serum of the patient, as compared with the serum and leukocytes of normal persons, is the one that falls most and rises most."

None of the authors quoted thus far has considered, or at least none has demonstrated, that this reaction between patient's leukocytes and patient's serum may be specific for the infecting bacterium in the particular case. The non-specificity for bacteria of variations in the phagocytic activity of patient's leukocytes is shown particularly

[1] *Aberdeen University Studies*, 1906, 21, p. 321.

[2] *Proc. Royal Soc.*, Lond., 1907, 80, B, p. 165.

[3] *Lancet*, 1908, p. 1073.

[4] *Comp. rend. de la Soc. Biol.*, 1908, 65, p. 480.

[5] *Ibid.*, 1909, 66, p. 930.

well in the work of Shattock and Dudgeon in which they used melanin in a large proportion of their experiments. And it is shown also, of course, in these cases of non-infectious disease studied by several authors. The fact that variation in the phagocytic activity of leukocytes in the presence of homologous serum in some cases may be specific for the infecting bacterium seems to have been first pointed out by Rosenow.[1] Rosenow found, in a case of pneumococcus endocarditis, that, when using the infecting strain, the opsonic index was lower when obtained with the patient's leukocytes than when normal leukocytes were used. But when a strain of pneumococcus obtained from a case of pneumonia was used, the index was slightly higher with the patient's own leukocytes than with normal leukocytes. In other cases of pneumococcus endocarditis, he failed to find such a specific relation. In one case he found that altho patient's leukocytes readily ingested bacteria either in their own or in normal serum, yet patient's leukocytes in patient's serum had no power to destroy pneumococci; patient's leukocytes in normal serum, however, or normal leukocytes in patient's serum readily destroyed the bacteria. This reaction was not specific for the infecting strain. But in a case of pseudodiphtheric cystitis, Rosenow[2] found the opsonic curve determined with the patient's leukocytes to be quite different from that obtained with normal leukocytes and to show a more favorable response to bacterial inoculations. The curve of the phagocytic power of the leukocytes also showed a favorable response on the part of the leukocytes to the inoculations.

Because of such variations in the leukocytes as well as in the serum, some observers have recommended that an index be obtained by using the whole blood from the patient, whole normal blood being the standard for comparison. This has been called the "hemophagocytic" index, the "opsono-cytophagic" index, and by other names. This is the method originally used by Leishman, and almost lost sight of after the introduction by Wright and Douglas of the opsonic index. The advantage of this index is that it takes into consideration all sorts of variations in the leukocytes as well as in the serum, but the presence of a leukocytosis would be expected to lower this

[1] *Jour. Infect. Dis.*, 1909, 6, p. 245.

[2] *Ibid.*, 1909, 6, p. 296.

index instead of raising it, for the larger number of leukocytes in the same volume of the mixture probably would ingest a smaller average number of bacteria. Glynn and Cox (*loc. cit.*) lay great stress on this index, which they obtain by suspending patient's washed leukocytes in the homologous serum and comparing them with normal leukocytes in normal serum. They assert that this index can be accurately calculated from separate determinations of the opsonic index and of the phagocytic power of the leukocytes (the "cytophagic" index). In my case this index could not have been so calculated.

My own work was carried out principally in connection with the following case of recurrent and complicated erysipelas.

A laborer, single, 32 years of age, had a first attack of erysipelas, facial type, in April, 1908; the second attack in October, 1908, was also facial, later migrating down the back. On leaving the hospital the left ear suddenly became painful; after a few hours the membrane ruptured, and a profuse discharge of pus occurred, which continued until May, 1909, when, following a recurrence, the discharge disappeared almost completely, and thenceforth remained but slight. The third attack was in December, 1908, beginning in the left ear and extending down the back. The patient remained in the hospital constantly from this attack until his death (May 20, 1909). During the next four weeks after this attack three relapses occurred. It was at this time that the patient came under observation, and I am indebted to Dr. Weaver and to Dr. Friedberg for the opportunity of studying the case in their wards at the Cook County Hospital. Following this last attack streptococcus inoculations were begun. Subsequently the patient had four recurrences and one relapse, making a total of six recurrences and four relapses. Two of these attacks began in the discharging ear, and all of the others either on the cheek or the nose. With the exception of the first, every attack assumed the migrating type. There was no history of nasal catarrh, or of sinus infection, tho the patient stated that during each attack of erysipelas there had been a moderate nasal discharge, and when first seen there was slight tenderness over both supramaxillary sinuses. The nasal mucus had been slightly blood-tinged for several years.

The pus from the ear contained a nearly pure culture of hemolyzing streptococci. Cultures were made on blood agar, killed with 25 per cent solution of galactose,[1] and on January 20, 50,000,000 cocci were injected. At this time the opsonic index, determined in the ordinary way, was 0.36, and the leukocyte count 11,950. The next day the index rose nearly to normal, and remained there until the sixth day, when another injection of 50,000,000 was given; the index now rose abruptly to 2.5, and then fell abruptly to 1.3, thence declining slowly to normal. A subsequent injection of 50,000,000 cocci produced no effect upon the index. Three days later 150,000,000 cocci were injected. No immediate effect was produced on the index, but the next day the patient showed indications of acute endocarditis; and the day after this he developed a multiple arthritis which lasted several days. Four days after this inoculation he received another inoculation of 400,000,000 cocci, and the index rose to 1.8. Two days later

[1] Weaver and Tunnicliff, *Jour. Infect. Dis.*, 1908, 5, p. 589.

his erysipelas recurred. Eight days later he had a relapse. A blood culture at this time was sterile. Two weeks later the mastoid process was opened, and a subdural abscess containing about an ounce of pus was drained. The general condition of the patient was apparently not improved by the operation.

Because of the fact that the phagocytic power of the patient's blood was considerably increased by the addition of a small amount of normal human serum, and was increased even more by normal horse serum (see Table 5), the patient was injected on March 14, 1909, with 10 c.c. of fresh normal horse serum.[1] This was five days after the mastoid operation, from the immediate effects of which he had quite recovered. He was feeling a little weak, but just about the same as before the operation. The injection was given about noon. In a short time he began to feel much better; that evening he ate a hearty supper, and sat up for a few hours—neither of which he had previously been able to do since his operation; that night he slept better than he had for a long time. The next morning he still declared that he felt better, but his erysipelas had recurred. The attack was a slight one, and he recovered completely from it in about five days. In view of the remarkable tho transitory improvement in the general condition, the recurrence was looked upon at that time as possibly a coincidence.

Two weeks later 500,000,000 streptococci were injected. There was no local reaction, but the next day there was moderately severe pain in the unaffected ear. A couple of days later there was considerable stiffness, and pain on motion in the muscles of the back of the neck. There was slight tenderness over the linea nuchae, but none over the mastoid. In the course of a week or so these disagreeable symptoms disappeared, and he seemed to be somewhat better. This was interpreted as being a positive reaction following a somewhat severe and prolonged negative phase. It is questionable if there was any net gain. About five weeks after the first injection of horse serum when the patient was in fair condition, 8 c.c. of fresh normal horse serum were injected into the right arm. The next day there was a rather severe local serum reaction and the temperature had risen somewhat. During the next four days the general condition became worse; the patient was weaker, the temperature reached 101° F., or 102° F. every day, being remittent in type, the pulse was accelerated, and the discharge from the mastoid became profuse and bloody. The cutaneous reaction extended, but was limited to the arm. The whole affair was probably a severe form of serum disease, tho it presented many points of similarity to a general septicemia. Finally, on the sixth day after the injection, there was a recurrence of the erysipelas, which promptly migrated down the entire back. This attack lasted six days, and then the mastoid discharge suddenly stopped, and the wound closed up almost completely. After that there was a slight mucous discharge but no pus. On the fourth day of the attack, the patient developed an acute pleurisy. The chest was twice aspirated and both times a thick fluid containing flocculi of fibrin was obtained. Unfortunately no smears or cultures were made.

[1] Therapeutic injections of normal horse serum have been tried and recommended by a number of observers, altho the rationale of this method of treatment is not clear. It is known, however, that the effects of a foreign serum may be quite different *in vivo* from what might be expected from experiments *in vitro*.

Cole and Smirnow (*Bull. Johns Hopkins Hosp.*, 1908, 19, p. 249) have shown that a property similar to that ascribed by Bail to his so-called "aggressins" may be present in normal serum. They noted that pigeons normally possess a surprising degree of resistance to B. pneumoniae. But pigeon serum, when injected into susceptible animals with a dose of living bacteria, instead of protecting them. rendered them more susceptible than controls. Rabbit serum produced a similar effect on mice.

It seemed clear from these two experiences that the horse serum was responsible for the recurrence in both instances. The delay in the second recurrence may have been due to some immunity having been acquired which postponed the attack for a few days, and it may be that the severe serum disease had the effect of reducing the patient's resistance and allowing the latent infection to assert itself.

Following this attack of erysipelas, altho the condition of the ear improved so remarkably, the patient's general condition became worse and in about two weeks he had another recurrence. He recovered completely from the erysipelas in about six days, but the fever remained, being intermittent in type, and reaching 103°–104° F. every day; four days later (May 20, 1909) he died.

An interesting phenomenon was observed during each of the last two attacks. About 24 hours before the appearance of the typical erysipelatous eruption, a patch of dull red was observed upon the nose. This was not the seat of any subjective sensations and was not in the least elevated or edematous. It did not have the tense, shiny appearance of a typical patch of erysipelas, nor the sharp contour. It was merely an indefinite erythematous patch. At the same time, or 24 hours earlier, the skin at the back of the neck and between the shoulders presented an edematous appearance. There was no distinct patch, there was no redness, there were no symptoms, and the edema itself was very much softer than that of an erysipelatous patch, pitting quite readily. The typical erysipelatous eruption occurred almost simultaneously in these two areas. These appearances could hardly be interpreted as a typical form of the eruption, for when the eruption came it was perfectly typical; perhaps they represent a true prodromal eruption.

Following the early inoculations a mild local reaction was usually obtained. It was never troublesome, and never lasted longer than two days. Later this reaction failed to appear. The leukocyte count showed great fluctuations (5,000 to 23,000) which did not seem to be related to the clinical condition, to the opsonic index, or to the inoculations. The percentage of polymorphonuclears remained close to 90 per cent, and late in the disease the stained specimen showed considerable variation in the size and staining properties of the leukocytes. No unusual types were seen. The hemoglobin early was 90 per cent; March 20 it was 57 per cent, and on May 14 it was 67 per cent (Sahli).

On February 9, the day before the third recurrence, and after the patient had received five inoculations, it was observed that the

index determined with the patient's leukocytes was much lower than when determined with normal leukocytes, that is to say, the patient's leukocytes, while acting as well as normal leukocytes in normal serum, showed but little phagocytosis in the homologous serum (see Table 1). This phenomenon was observed repeatedly. Table 1 shows the phagocytic indices obtained in the various combinations made by using the leukocytes and serum from a normal person, from this case—designated in the table as M—and from a case of convalescent erysipelas. The suspensions of leukocytes in this and the succeeding experiments were of practically equal density. The following method of standardization was used: Sus-

TABLE 1.

Phagocytic indices obtained by using serum and leukocytes from one normal person and from two cases of erysipelas, the leukocytic suspensions being of equal density.

Leukocytes	Serum		
	Normal	Convalescent Erysipelas	Case M
Normal	3.13	3.20	5.5 (index 1.76)
Convalescent erysipelas	3.6	3.07	5.72 (" 1.59)
Case M	3.56	4.98	0.76 (" 0.21)

pensions of leukocytes were first made from the "cream" of the washed and centrifuged specimens of the different bloods to be compared, in the ordinary way. Then by means of a high power lens and the ruled stage, the number of polymorphonuclear leukocytes in a given volume of each suspension was determined. The stronger suspensions were then diluted until they were of the same density as the weakest.

This table shows that tho the index of the patient's serum (Case M) when obtained with normal leukocytes or with the other patient's leukocytes is high, 1.76 and 1.59, the index obtained with this patient's own leukocytes is very low, 0.21. The fact that the patient's leukocytes acting in other serums may give even a greater degree of phagocytosis than normal leukocytes, shows clearly that the fault lies not with the leukocytes but with the serum. This may be due to one of two things or to both: either some element necessary to phagocytosis, and present in normal serum, is largely absent in this patient's serum, or else there is an inhibitory substance present

which is specific for the patient's leukocytes. The evidence on this point is not conclusive, but the next two tables show some figures which tend to support the first view.

TABLE 2.

Phagocytic and opsonic indices obtained by using normal and patient's serum, and suspensions of normal and of patient's leukocytes, and of patient's leukocytes which have been "soaked" in normal serum for one hour in the incubator, and then washed.

Leukocytes	Phagocytic Index		Opsonic Index
	Normal Serum	Patient's Serum	
Normal	10.94	4.92	0.45
Patient's	16.04	1.63	0.10
Patient's treated with normal serum	24.88	10.43	0.42

TABLE 3.

Showing the increase in phagocytic index obtained by adding a small amount of normal serum to patient's serum, and comparing this index with that obtained with normal leukocytes and normal serum.

Leukocytes	Serum	Phagocytic Index
Patient's	Patient's	1.63
"	" +1/20 vol. normal serum	11.72
"	NaCl + " " " "	1.78
Normal	N.	10.94

NOTE.—The phagocytic mixtures in these experiments consisted usually of 1 volume of serum, 1 volume of suspension of leukocytes, and 1 volume of suspension of bacteria. In this and succeeding tables where the term "1/20 vol." is used, it means 1 volume of serum which has been diluted in the ratio of 1:19 with normal salt solution.

In Table 2 the index of the patient's serum when determined with normal leukocytes is 0.45 and when determined with the patient's own leukocytes is only 0.10. But when the patient's leukocytes are first "soaked" in normal serum for one hour in the incubator, and washed before using, the index is practically the same as when normal leukocytes are used. In Table 3 it is seen that when a small volume of normal serum is added to the patient's serum, the opsonic power of the patient's serum (with respect to patient's leukocytes) is enormously increased. This would seem to indicate that there is some substance necessary to phagocytosis present in normal serum, which is largely or wholly lacking in this patient's serum and that it need be present only in small amount because the opsonic power of a given volume of the patient's serum may be considerably increased by the addition of so much of this substance as is contained in 1/20 of that volume of normal serum. Moreover, this substance may be taken up by the patient's leukocytes when exposed to normal serum

and so bound as to resist repeated washing. Whether this substance is taken up mechanically by the leukocytes, or whether there is a firmer union, cannot be stated definitely.

It would be natural to suppose that this substance which is lacking is complement, or at least a complement-like body, for the patient's fresh serum acts much like the heated serum of those individuals whose sera show the phenomenon of reactivability, but this is not necessarily true, for this patient's serum is not reactivable, as the following table shows:

TABLE 4.

Sera which activate the patient's fresh serum will not reactivate the heated serum. Patient's leukocytes only are used.

Serum	Phagocytic Index
Patient's	0.94
" +1/20 vol. normal serum	5.54
" +1/20 " serum "S"	4.36
" (heated)	0.50
" " +1/20 vol. patient's serum	0.33
" " +1/20 " normal "	0.30
" " +1/20 " serum "S"	0.14
Normal serum diluted 1/20	0.20
Serum "S" diluted 1/20	0.35

These figures show that the patient's heated serum is not reactivated by a small volume of patient's fresh serum nor by a small volume of either of two other sera, tho these same sera are able to activate the unheated serum.

This does not mean definitely that the activating substance is not complement, for there is still the possibility that lack of reactivability in a given serum is due, not to the lack of thermostable substance, but to an inhibition produced by the heating. In fact the figures of Table 4 might almost be urged in support of that view.

The effect of heat and age on the power of normal serum to activate this patient's serum is shown in Table 5.

TABLE 5.

Old or heated serum possesses less reactivating power than fresh serum.

Leukocytes	Serum	Phagocytic Index
Patient's	Patient's	0.94
"	" +1/20 vol. normal serum	5.54
"	" +1/20 " " " (heated)	2.12
"	" +1/20 " " " (old)	1.72
"	" +1/20 " " horse serum	7.60
"	" +1/20 " " " " (old)	1.48
"	$\frac{1}{20}$ vol. normal serum	0.20
"	$\frac{1}{20}$ " horse "	0.22

The "old" human serum used here was six days old, and the "old" horse serum four months old; both had been kept in the ice-box.

These figures show that old serum and heated serum (60° for 30 m.) lose much but not all of their power of activation. Incidentally it is seen that normal horse serum has a greater activating effect than normal human serum and hence its injection into the patient.

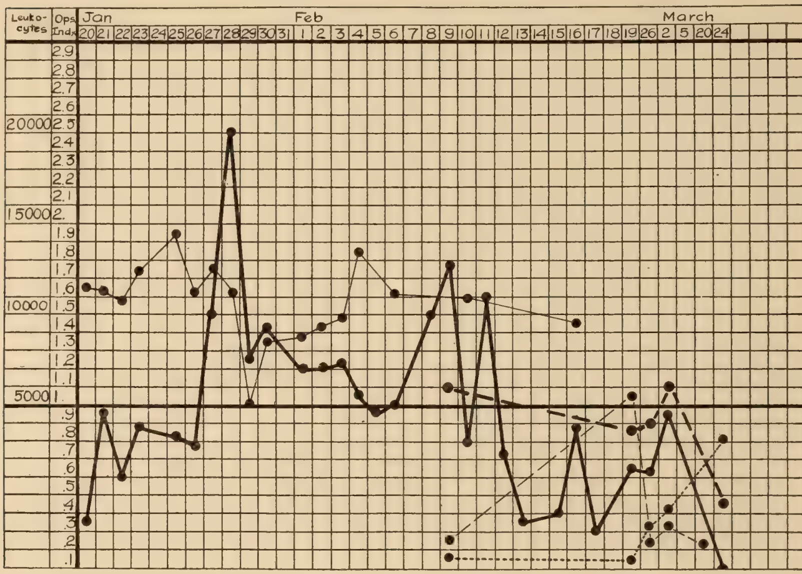

CHART I.—Solid heavy line=opsonic index obtained in the usual way with normal leukocytes.
Fine broken line=opsonic index obtained in the usual way with the patient's leukocytes.
Heavy broken line=phagocytic power of the patient's leukocytes in normal serum as compared with the phagocytic power of normal leukocytes in normal serum.
Dotted line=phagocytic power of the patient's leukocytes in the patient's serum as compared with the phagocytic power of normal leukocytes in the patient's serum.
Solid fine line=leukocytic curve.

It has been suggested that possibly the low opsonic power of this patient's serum with respect to his own leukocytes might be due to an inhibitory substance which is neutralized by normal serum and which either does not affect normal leukocytes or is neutralized by them. I am able to present very little direct evidence in favor of such a view, but everything that has been said so far in favor of the opposite theory might be explained equally well by this one. Results

have been obtained a few times which suggest such an inhibitory action, but the differences obtained have never been marked. On the other hand, normal serum appeared at times to exert an inhibitory action on the patient's serum. In another case (S) using patient's leukocytes and patient's serum, the phagocytic index was 1.91; when 1/20 volume of normal serum was added the index was reduced to 0.62.

Results have been obtained which indicate variations in the phagocytic power of the leukocytes, independent of changes in the serum. In many of the experiments the patient's leukocytes were practically normal when in normal serum, but sometimes the leukocytes were more active than normal, and on two occasions they were less active than normal leukocytes when in normal serum (once slightly so, and once markedly so).

In Table 1 the patient's leukocytes (Case M) are seen to be more active than normal leukocytes when in another patient's serum tho that serum has a normal index, yet in the homologous serum they show very little phagocytosis. In Table 2 it is seen that the patient's leukocytes when in normal serum are considerably more active than normal leukocytes, altho the index of the patient's serum is low (0.45). In Table 6 are shown results obtained by using the infecting and one other streptococcus in various combinations of serum and leukocytes.

TABLE 6.

The Apparent Specific Relationship between Leukocytes, Serum, and Streptococci in Case M.

Leukocytes	Phagocytic Index		Opsonic Index	
	Normal Serum	Patient's Serum		
Normal........	4.32	1.25	0.30	Infecting streptococcus
Patient's.......	2.52	1.85	0.73	
Normal........	9.73	6.42	0.66	Laboratory streptococcus
Patient's.......	8.26	8.82	1.06	

These figures were obtained on the day that the patient was recovering from a relapse, and so it might be expected that the defensive powers would be at their height. In both instances the opsonic index of the patient's serum obtained by using the patient's leukocytes is higher than that obtained by using normal leukocytes; in

both instances the index is lower with the infecting streptococcus than with another strain. In the case of the infecting streptococcus, the patient's own leukocytes in normal serum are less active than normal leukocytes, altho in their own serum they are fully as active as, or possibly more active than, normal leukocytes. In the case of the stock streptococcus the patient's leukocytes in normal serum are practically normal, and when in their own serum are even more active than normal leukocytes. A comparison of these figures suggests an appreciable degree of specificity in the triple relations of serum, leukocytes, and streptococci. From these figures it is difficult to say wherein this specificity resides, but it seems almost certainly to involve both serum and leukocytes; for it is shown by the leukocytes in normal serum and by the serum when using normal leukocytes. These figures assume all the more significance in view of the general uniformity in the behavior of any given serum toward various strains of streptoccoci grown under similar conditions. In the following experiment the results show that these leukocytes may behave differently toward different streptococci in the same normal serum:

TABLE 7.

Leukocytes	Phagocytic Index		Opsonic Index	
	Normal Serum	Patient's Serum		
Normal........	6.0	6.16	1.00	Infecting streptococcus
Patient's.......	4.0	1.43	0.36	
Normal........	8.0	4.88	0.61	Laboratory streptococcus
Patient's.......	7.22	1.56	0.51	

On this occasion the index was higher for the infecting strain and was lower when obtained with the patient's leukocytes (the exact reverse of the conditions in the previous experiment), but one relation remains the same, viz., while the patient's leukocytes in normal serum are practically normal for the stock streptococcus, they are somewhat less active than normal for the infecting strain. The difference is slight but suggestive.

The deficiency of the patient's leukocytes is more clearly shown in this experiment in which the infecting streptococcus was used. Here, altho the patient's leukocytes are practically normal in his

own serum, they are much less active than normal in normal serum, and consequently the index is slightly higher with patient's leukocytes than with normal leukocytes:

TABLE 8.

Leukocytes	Phagocytic Index		Opsonic Index
	Normal Serum	Patient's Serum	
Normal........	12.20	1.16	0.095
Patient's......	6.80	0.94	0.14

In an attempt to throw some light on the apparent discrepancy between the action of horse serum *in vivo* and *in vitro*, the phagocytic power of serum and leukocytes was tested with respect to the second generation of a recently isolated strain of a hemolytic streptococcus obtained from the patient's ear, grown on blood agar, and kept in the ice-box, and with respect to a strain also obtained from the patient, and grown on plain agar in the laboratory for three months.

TABLE 9.

The Difference in the Opsonic Index for Different Strains of Streptococci from the Patient.

Leukocytes	Serum	Phagocytic Index		
Normal	Normal	6.16		
Patient	Patient	3.87	0.62	Hemophagocytic index for long-cultivated strain
"	" +1/20 vol. normal serum	6.48	0.95	
Normal	Normal	6.88		
"	Patient	12.75	1.90	Opsonic index for recently isolated strain
Patient	Normal	6.01		
"	Patient	12.18	2.03	
"	" +1/20 vol. normal serum	6.81	1.13	

The interesting fact is here shown that while the index is low for the strain that has been cultivated for three months (0.62), it is high for the strain recently isolated (1.9). This index is practically the same whether determined with the patient's leukocytes or with normal leukocytes. Furthermore, the addition of a small amount of normal serum to the patient's serum raised the index for the former (from 0.62 to 0.95), while it lowers the index for the latter strain (from 2.03 to 1.13). This might be urged to be an adequate explanation for the bad results obtained by injection of the patient with fresh horse serum.

Several other cases of relapsing and of migrating erysipelas were

tested for the possible presence of peculiar relations between serum and leukocytes similar to those found in this case. The results of this investigation are reported elsewhere.[1]

SUMMARY.

The principal observations in this case may be briefly summarized as follows:

Inoculations of the infecting streptococci killed by galactose solution, in doses of 50,000,000 to 500,000,000, were of doubtful benefit. Early in the course of the disease the opsonic index was raised, but no considerable improvement in the local or general condition can be ascribed to the inoculations. The results of the phagocytic experiments indicate that there may be a certain degree of specificity in the relations of serum leukocytes and microbes (in this case streptococci) to one another. This specificity for the infecting streptococci was shown in this case by the serum independently of the leukocytes and by the leukocytes independently of the serum, but it was most marked when leukocytes were suspended in their own serum. Moreover, there was a specific relation between the patient's serum and leukocytes which was different for different strains of streptococci. At times the opsonic index obtained with the patient's leukocytes was much lower than when obtained with normal leukocytes. This was owing either to the lack in the serum of some element necessary to phagocytosis which is present in normal serum (and in normal leukocytes), or to some inhibiting substance specific for the patient's leukocytes and neutralized by normal serum. When a small amount of normal serum (1/20 volume) was added to the patient's serum, or if the patient's leukocytes were first "soaked" in normal serum and then washed, the index determined with the patient's leukocytes was practically the same as when determined with normal leukocytes. When the serum was heated it lost much of its opsonic power and could not be reactivated by sera that would activate it in the unheated state. The fact that the patient's leukocytes gave a lower opsonic index than normal leukocytes has sometimes been due to an increased activity of the patient's leukocytes in a foreign serum while normal in their own. Sometimes a higher index was obtained with the patient's leukocytes than with normal

[1] *Trans. Chi. Path. Soc.*, 1909, 8.

leukocytes and this might be explained by a greater activity of the leukocytes in their own serum, a decreased activity in normal serum, or by both of these factors. At times the patient's leukocytes were less active than normal leukocytes in normal serum while in their own serum the difference was less marked. The serum, then, showed considerable variations in opsonic power either above or below normal independent of the conditions of the leukocytes, and the leukocytes may show variations in phagocytic power dependent however, to some extent at least, on the condition of the serum. When the patient's leukocytes were normal in phagocytic power in normal serum the patient's serum was either above or below normal in opsonic power when tested with normal leukocytes. When the patient's leukocytes were below normal the serum was either normal or below—never above. When leukocytes were above normal (one instance) the serum was also above normal. When the leukocytes tested in their own serum were normal (one instance) the serum tested with normal leukocytes was above normal. When the leukocytes in their own serum were above normal the serum was below normal. When the leukocytes in their own serum were below normal the serum was either above or below normal. The changes in the serum appeared earlier and were more persistent, more irregular, and more marked than were the changes in the leukocytes. The opsonic index was sometimes higher, sometimes lower for the infecting than for the laboratory streptococcus; and for a streptococcus strain recently isolated from the patient the index was found to be much higher than for a strain also obtained from the patient but cultivated for a considerable length of time. In connection with these observations it may be pointed out that the opsonic index as ordinarily determined in reality only measures the amount of opsonin present with respect to a given bacterium and with respect to normal leukocytes; in view of the observations here recorded the opsonic index consequently cannot be regarded as a reliable indication of the phagocytic power of the patient's blood. The general effect of the opsonic variations here described is to limit the significance of the opsonic index. It is noteworthy that so far specific variations in the behavior of serum and leukocytes with respect to the infecting bacterium have been observed only in cases of chronic infection,

and it may be that it is only in long-standing infections that such special adaptations on the part of both microbes and host have time to develop. Finally it should be noted that injections of fresh normal horse serum seem to have a bad effect in this case. Possibly this was owing to the fact that while fresh horse serum increased greatly the opsonic power of the patient's serum for a streptococcal strain long cultivatd outside of the body, it seemed to lower the opsonic power for a recently isolated strain. Naturally it would be highly desirable to study the action of the injection of human serum in similar cases.

CONCLUSIONS.

The inoculation treatment was without benefit in a case of long-standing recurrent and relapsing erysipelas.

There may be an appreciable degree of specificity in the relation of serum, leukocytes, and bacteria, to each other. The serum may show specific peculiarities with respect to both leukocytes and bacteria, and the leukocytes may show specific peculiarities for serum and bacteria.

The phagocytic power of leukocytes may differ when in their own serum and when in normal serum. Variations in the opsonic power of the serum may occur independently of variations of the phagocytic power of the leukocytes but such variation on the part of the leukocytes, however, is not entirely independent of the variations of the serum. The phagocytic power of the leukocytes may be above or below normal in their own or in a foreign serum. The changes in the serum may appear earlier and be more persistent and more marked than the changes in the leukocytes.

The low phagocytic power of leukocytes in their own serum may depend either upon the lack of some element necessary to phagocytosis or to some inhibiting substance specific for the leukocytes in question and neutralized by normal serum.

The addition of a small amount of normal human or horse serum may increase the opsonic power of the blood; this activation was in this case quite different from the reactivation of heated serum. Injections of fresh horse serum may seem to have unfavorable results, possibly because it interferes with the phagocytosis of the infecting organism.

The opsonic index may be higher for a freshly isolated strain of the infecting streptococcus than for a long-cultivated strain.

A STUDY OF THE CONCENTRATION OF THE ANTI-BODIES IN THE BODY FLUIDS OF NORMAL AND IMMUNE ANIMALS.*

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The presence of antibodies of various kinds in serum has long been known, and the concentration in that fluid has been carefully studied. The presence of antibodies in the various other body fluids has not been so carefully investigated, nor has sufficient allowance been made for individual variations in animals of the same species. While the authors were associated with Dr. Carlson in his work on lymph formation, he suggested that a careful comparison between the concentration of the antibodies in the various body fluids of the same animal might be of considerable importance in determining the differences between the lymph and serum, and in that way throw light on some of the problems of lymph formation, and possibly also on the point of origin of antibodies. When the work was begun, we intended to collect lymph from the different organs, but the practical difficulties encountered in introducing cannulae into the delicate lymphatics of such organs as the spleen was so great, that the project was temporarily abandoned, and the work has been confined to a comparison between serum, lymph from the cervical lymphatics, lymph from the thoracic duct, pericardial fluid, cerebrospinal fluid, and aqueous humor. Thus far work has been done on the hemolysins, hemagglutinins, agglutinins for the typhoid bacillus, the protein precipitins, and the opsonins, bacterial and erythrocytic. No work has yet been undertaken on the bacteriolysins. We have not enough data to enable us to draw any broad conclusions, and we will content ourselves with presenting what we believe to be the facts under the various conditions studied.

Literature.—The first studies that we were able to find on the relative concentration of antibodies in the various body fluids were those of Pegano,[36] who found the concentration of hemolysins of the thoracic lymph in dogs lower than that of the serum. Falloise [11] and later Batelli[4] confirmed the work of Pegano. Hughes and Carlson [8] working on normal dogs, horses, and cats found the concentration of hemolysins for

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rabbit corpuscles in the body fluids to form a descending series: serum, thoracic lymph, neck lymph, pericardial fluid, aqueous humor. No lysins were found in the cerebrospinal fluid. Straus and Wolf[40] studied the hemolytic power against rabbit corpuscles of the cerebrospinal fluid, edema fluid, pleural and pericardial transudates, and blister fluid, and attempted to correlate the hemolytic strength with the protein content. Marshall and Morgenroth[25] found anti-complement and anti-amboceptor in a pathological exudate—an ascites fluid. Hedinger[15] studied the hemolytic power of non-inflammatory exudates like those arising from cirrhosis of the liver and heart failure, and found that they were not so hemolytic as the serum. The inflammatory exudates arising from cases of tuberculosis and carcinoma were not so strongly hemolytic as non-inflammatory exudates. He failed to find hemolysins in the fluid from an ovarian cyst, or in the cerebrospinal fluid in two cases of tuberculosis. Marshall[24] found that pleural and ascites fluids were more strongly hemolytic than the serum from an infant. But no conclusions can be drawn from this comparison in regard to the comparative hemolytic power of serum and other body fluids in the same individual. He found a multiplicity of amboceptors and complements in the fluids that he studied. Grollo[14] could find no amboceptors for rabbit corpuscles in transudates, but found them in exudates, altho in the latter complement is often lacking. He suggests this method as a means of diagnosis between transudates and exudates. Lüdke[22] confirmed the findings of Marshall in regard to the hemolytic strength of transudates and exudates. Granström[13] found wide variations in the hemolytic content of transudates and exudates, and could establish no characteristics essential for either. The hemolysins did not run parallel with those of the blood. Isolysins are found less frequently in transudates and exudates than in the blood. Hemolysins were not found in the cerebrospinal fluid. Isolysins and heterolysins were found independent of the albumen content, number of the leukocytes, and the osmotic pressure of the fluids tested. Tedeschi[41] found precipitins in both transudates and exudates, less frequently in the latter than in the former. Mioni[30] found amboceptor but no complement for guinea-pig corpuscles in the pericardial fluid of the ox. Bard[2] claims to have found hemolysins in the cerebrospinal fluid of patients, and found that they were increased during various diseases. Massaglia[26] could not confirm the work of Bard. His results in both healthy and diseased individuals were negative. The presence of antibodies for syphilitic material in the cerebrospinal fluid has been shown by various investigators, among them Morgenroth and Stertz,[31] and Wassermann and Plaut.[42] Gatti[2] could demonstrate no hemolysins in the aqueous humor of the ox. Levaditi[21] showed that there is normally no opsonin in the aqueous humor; but if the fluid of the anterior chamber of the eye of an immune animal is withdrawn, the newly formed aqueous humor will contain opsonin. Böhme[6] investigated the opsonin content of pleural, peritoneal, and abscess fluids. He found that usually in such cases the opsonin content of the fluid was reduced for the infecting organism, but remained unchanged for other bacteria. He could find no opsonin in normal cerebrospinal fluid, but found them there after an inflammation had been set up in the dura. He could not develop opsonins in the cerebrospinal fluid by repeated puncture as Levaditi had done by drawing off the aqueous humor. He believes that there is a relation between the protein content and the opsonin action of a fluid.

Methods.—The plan of study adopted was to determine first the concentration of the antibodies in the body fluids of normal cats and dogs; then the concentration in actively immunized animals; and, finally, to study the passage of the antibodies from

the blood into the other body fluids in animals passively immunized by the withdrawal of large quantities of blood, and the injection of a corresponding amount of warm, defibrinated blood from an actively immunized animal.

The body fluids were secured under as nearly aseptic conditions as possible. The animal was anesthetized with ether, and kept in a state of complete anesthesia, by the administration of the vapor through a trachea cannula or through a tube introduced through the larynx. The neck lymphatics were then isolated, and small, sterile, glass cannulae provided with sterile, rubber tubing were inserted. If there was no free flow of lymph, the neck was gently massaged. The lymph was never allowed to come in contact with the air of the room, for as soon as it filled the cannula and a part of the rubber tubing, it was drawn off by means of a fine, sterile Pasteur pipette, and placed in a dry, sterile test tube plugged as for bacteriological work. The lymph was allowed to coagulate spontaneously in the test tube, and was then defibrinated, and the delicate coagulum removed.

The thoracic duct was tied off at the same time as the isolation of the neck lymphatics so that the lymph formed during the experiment was retained in the duct. Usually the lymph from this duct was not collected until the animal had been bled to death, altho sometimes it was collected simultaneously with the neck lymph. The routine method was to draw the lymph by means of a Pasteur pipette provided with a bulb, so that the fluid never came in contact with the air at all. This fluid was also defibrinated.

The pericardial fluid was never collected until after the death of the animal by very complete bleeding from the arteries and veins of the neck. The thorax was opened by removing the sternum, a small hole was cut into the pericardium, and the fluid was removed by means of a sterile Pasteur pipette.

We found it a good plan in our experiments to suspend the animal by the jaws for a few minutes before attempting to withdraw the cerebrospinal fluid. This drained away the blood from the head and made admixture of this fluid with blood less likely. Our method was to open the dura between the first and the second cervical vertebrae, and then remove the fluid by means of the Pasteur pipette. The end of the pipette must be well rounded in the flame, otherwise rupture of the delicate blood vessels of the meninges is likely to follow.

The method of collecting the aqueous humor was simple and easy. It consisted in thrusting a sharp pointed Pasteur pipette into the anterior chamber of the eye through the cornea, and allowing the aqueous humor to flow into it, largely by the tension within the eyeball. A little suction sufficed to remove the last drop of the fluid.

The serum was secured from blood drawn when the animal was bled to death, and in most cases was freed from the corpuscles at once.

Careful notes were made in regard to the condition of the fluids, and in most cases where there was any admixture of blood, the fluid was discarded.

HEMOLYSIS AND HEMAGGLUTININS.

Methods.—The study of the hemolysins and hemagglutinins in normal* animals was made on dogs only. The animals utilized were brought in from various parts of the city. Most of the tests were made with rabbit corpuscles in 5 per cent suspension in 0.9 per cent NaCl solution. In some cases rat and horse corpuscles were used.

* The term "normal" means animals which had not previously been immunized by us. We had no way of knowing what their history had been previous to coming to the laboratory.

Our methods were the following: Quantities of the various body fluids of the animal to be tested varying between 0.1 c.c. and 0.0001 c.c. were placed in a series of eight dry, sterile test tubes plugged as for bacteriological work. In order to make the necessary measurements with a pipette graded to $\frac{1}{100}$ of a c.c., dilutions of the body fluids $\frac{1}{10}$ and $\frac{1}{100}$ were made. To the fluid in each tube enough sterile 0.9 per cent NaCl solution was added to make the total volume up to 0.4 c.c. To this was then added 0.2 c.c. of a 5 per cent suspension of the corpuscles to be tested. In this way we got dilutions of the fluid varying between 1:6 and 1:6,144. All of the fluids from the same animal were prepared, the tubes were placed in a block containing a suitable number of holes, and adjusted to the sliding platform of a shaker in an incubator warmed to 37° C. The shaker was run by water power, and the motion was rapid enough to secure constant, thorough agitation, but not violent enough to injure the corpuscles. The routine technic was to keep the tubes in the shaker for an hour and in the ice-box from 12 to 20 hours to permit sedimentation of the corpuscles before the final reading.

In determining the amount of hemolysis in the final reading, the following method was employed: A measured sample of the corpuscle suspension in the test was sedimented in the centrifuge, and the supernatant liquid drawn off with a pipette. The corpuscles were then laked by adding distilled water to restore the original volume. This sample contained three times as much hemoglobin as the hemolytic tests, because 0.2 c.c. of the corpuscles were added to 0.4 c.c. of the fluid tested. Therefore, the above sample was diluted to three times its volume with water. This, then, would give exactly the same concentration of hemoglobin as in any tube in the test, provided that the hemolysis was complete, and is termed 100 per cent for this sample of corpuscles. By further dilution tubes containing 90 per cent, 80 per cent, etc., were prepared. No attempt was made to estimate closer than 10 per cent. A new scale was made for each sample of corpuscles.

The agglutinins were read from the same tubes as the hemolysins. The method employed to determine whether or not agglutination had occurred, was inspection of the rim of sedimented corpuscles. When the corpuscles, after sedimentation by standing in the ice-box, show a perfectly smooth, knife-edged border, no agglutination has occurred. If the border is slightly or decidedly roughened, agglutination has occurred At first this method was carefully supplemented by microscopic examination, but it was soon found so accurate that in the later experiments we depended entirely upon the observation of the rim of the corpuscles, and dispensed with the use of the microscope.

A. Normal animals.—The concentration of lysins and hemagglutinins in the body fluids of normal animals varies within rather narrow limits. This variation is great enough, however, to make it necessary that the comparison be made between the body fluids of the same animal. The following experiment shows the behavior of the body fluids of the normal dog.

Table I shows that the concentration of hemolysins is greater in the serum than in the other body fluids; thoracic lymph is next, at least in the case of rabbit corpuscles; and neck lymph is third.

This difference in the hemolytic power of serum or other body fluid against the corpuscles of different species of animals has been explained by Ehrlich and his coworkers on the basis of a multiplicity of amboceptors and complements, some of which are specific, some

TABLE 1.

COMPARATIVE HEMOLYTIC AND AGGLUTINATING POWER OF THE BODY FLUIDS OF A NORMAL DOG ON RABBIT AND RAT CORPUSCLES.

Dilution	Serum				Neck Lymph				Thoracic Lymph			
	Rabbit		Rat		Rabbit		Rat		Rabbit		Rat	
	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.
1:6	100	—	10	+	5	+	0	0	40	+	0	+
1:12	40	+	0	0	0	+	0	0	0	+	0	+
1:24	0	+	0	0	0	0	0	0	0	+	0	+
1:48	0	0	0	0	0	0	0	0	0	+	sp	—
1:96	0	0	sp*	—	0	0	sp	—	0	0	0	0
1:384	0	0	0	0	0	0	0	0	0	0	0	0

Dilution	Pericardial Fluid				Cerebrospinal Fluid				Aqueous Humor			
	Rabbit		Rat		Rabbit		Rat		Rabbit		Rat	
	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.
1:6	0	+	0	0	0	0	0	0	0	0	sp	—
1:12	0	+	0	0	0	0	sp	—	0	0	sp	—
1:24	0	0	sp	—	0	0	0	0	0	0	0	0
1:48	0	0	0	0	0	0	0	0	0	0	sp	—
1:96	0	0	0	0	0	0	sp	—	0	0	0	0
1:384	0	0	0	0	0	0	0	0	0	0	0	0

*It will be noted that in this table several tubes are marked "sp." By that symbol is meant hemolysis not due to the ordinary hemolysins. The appearance of the partially laked corpuscles is entirely different from that in the ordinary hemolytic test. The hemoglobin can be seen diffusing from the sedimented corpuscles, while the supernatant fluid remains perfectly clear. The hemoglobin has the peculiar reddish purple tint of reduced hemoglobin, instead of the clear red of oxyhemoglobin. Furthermore, laking may appear anywhere in the series, frequently, where no hemolysis is to be expected, and is met more often in fluids like the cerebrospinal, or aqueous humor, which are normally not hemolytic, than in the other fluids. Rat corpuscles seem more susceptible to this form of hemolysis than rabbit corpuscles. Complement seems to inhibit this form of hemolysis.

non-specific. In most cases the thoracic lymph of normal dogs is hemolytic for rat corpuscles, altho in the experiment cited above, such was not the case.

As may be seen from the table above, the concentration of agglutinins may be higher in the thoracic lymph than in the serum. Such, however, is not the usual finding. In 10 experiments on normal dogs we found in seven the concentration of agglutinins highest in the serum; in two it was highest in the thoracic lymph; and in one

the concentration was the same in both. The fact that the concentration of agglutinins may be greater in the thoracic lymph than in the serum, renders it hard to see how these antibodies can come from the blood by pure filtration, for in that case, we should expect the hemolysins to run a parallel course—a thing which they do not do—or else we must assume that the agglutinins pass through membranes more readily than the hemolysins. It would be necessary, also, on the basis of filtration, to assume sudden great changes in the concentration of the agglutinins in the blood, for on no other basis could we explain the fact that the concentration of agglutinins would be so much lower in the serum by the time the lymph reached the upper end of the thoracic duct, than it was at the time the lymph was formed. Of course other explanations are possible: there may be an active secretion of the agglutinins into the lymph from the blood, or the agglutinins, after being formed in the area drained by the thoracic duct, are thrown into the lymph, reaching the blood by that route. Much more investigation must be made before any conclusion can be reached on this point.

The pericardial fluid when collected under the best conditions never shows hemolysins for rabbit corpuscles. Agglutinins may or may not be present. In four of our ten supposedly normal dogs hemolysis was noted, in only one case amounting to more than 10 per cent. Of these four animals, two were in poor condition, emaciated, and generally run down, and both these dogs yielded excessive amounts of pericardial fluid; in the other two cases, the pericardial was found to contain a few erythrocytes. Agglutinins were found in all four of these cases and in three others, making a total of seven in ten. From these experiments we are inclined to believe that hemolysins are not found in the pericardial fluid of normal dogs. The fact that some animals showed hemolysins in the pericardial fluid we would explain as a pericardial transudate in two cases, and to admixture with blood in two cases. We did not test whether it was amboceptor, or complement, or both which was absent from the fluid, altho we have evidence on this point in immune animals. Agglutinins for rabbit corpuscles may or may not be present in the pericardial fluid of normal dogs.

As will be seen from Table 1 the cerebrospinal fluid and aqueous

humor of normal animals contain no lysins or agglutinins for rat or rabbit corpuscles. In our 10 experiments on normal animals there were no traces of hemolysis or agglutination in a single case where admixture of blood was eliminated. Our results with cerebrospinal fluid confirm those of Massaglia, who could find no lysins in that fluid, and are contrary to those of Bard, who claims to have demonstrated them there.

Conclusions.—1. In the normal dog hemolysins for rabbit corpuscles are found in the serum, neck lymph, and thoracic lymph, but are absent from the pericardial fluid, cerebrospinal fluid, and aqueous humor. They are most concentrated in the serum, less concentrated in the thoracic lymph, and are found only in traces in the neck lymph.

2. Agglutinins are found in the serum, neck lymph, and thoracic lymph of normal dogs. They may or may not be present in the pericardial fluid, and are always absent from cerebrospinal fluid and aqueous humor. In most cases the concentration descends in the following order: serum, thoracic lymph, neck lymph, pericardial fluid; altho in some cases, the order is thoracic lymph, serum, neck lymph, pericardial fluid.

3. Serum and thoracic lymph show a weaker hemolysis and agglutination toward rat than toward rabbit corpuscles. Neck lymph lakes and agglutinates rabbit but not rat corpuscles. Pericardial fluid agglutinates rabbit but not rat corpuscles. Cerebrospinal fluid and aqueous humor neither lake nor agglutinate rat or rabbit corpuscles.

B. Immunized animals.—Various methods of producing active immunity were employed with good success. It is of interest to ascertain what methods of immunizing yield the best results. We employed the following: (1) Immunization of dogs with rabbit blood: (*a*) intraperitoneally by a single large injection of from 80 to 150 c.c. of blood, (*b*) intraperitoneally by repeated small injections, (*c*) subcutaneously by repeated small injections. (2) Immunization of dogs with horse serum:* (*a*) by a single large intraperitoneal injection of 100–150 c.c., (*b*) by repeated, small intraperitoneal injections, (*c*) by

* This serum was secured aseptically, November, 1908, by drawing the blood from the carotid of a horse into jars. It was allowed to coagulate and stand in the ice-box until the serum came out. The serum was then sealed into bulbs and kept in the ice-box until used.

repeated, small subcutaneous injections. A comparison of the results secured by using the fluids directly from the animal, so far as the lysins are concerned, is not conclusive, for, as will be seen, complement is not increased, at least not in proportion to the amboceptors, if at all. This fact necessitates the use of sufficient complement to supply all the amboceptors present to demonstrate the true state of affairs. So far as the agglutinins are concerned, apparently a single large dose of the serum or blood may develop them more markedly than the other methods tried. The repeated, small intraperitoneal injections yielded the most uniform results.

As has been noted by numerous investigators, the increase in complement does not keep pace with the increase in amboceptors. The apparent increase in the hemolysins and in the agglutinins for rabbit corpuscles is shown in Table 2.

TABLE 2.

LYTIC AND AGGLUTINATING ACTION OF THE BODY FLUIDS OF A DOG IMMUNIZED WITH RABBIT BLOOD. (November 25, December 15, 10 c.c. rabbit blood intraperitoneally; December 15, 15 c.c. Fluids collected December 23.)

Dilution	Serum		Neck Lymph		Thoracic Lymph		Pericardial Fluid		Cerebrospinal Fluid		Aqueous Humor	
	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.
1:1½	—	—	—	—	—	—	0	+	0	0	0	0
1:3	—	—	—	—	—	—	0	+	0	0	0	0
1:6	100	+	60	+	100	+	0	+	0	0	0	0
1:12	100	+	0	+	60	+	0	+	0	0	0	0
1:24	50	+	0	+	0	+	..	..	..	..	..	..
1:48	0	+	0	+	0	+	..	..	..	..	..	..
1:96	0	+	0	+	0	+	..	..	..	..	..	..
1:384	0	+	0	+	0	+	..	..	..	..	..	..
1:1,536	0	+	0	0	0	0	..	..	..	..	..	..
1:6,144	0	0	0	0	0	0	..	..	..	..	..	..

From Table 2 it can be seen by comparison with Table 1 that the repeated injections of rabbit blood does not appear to increase to any very marked extent the hemolytic power of the body fluids over that of a normal animal.

There is, however, a marked increase in the power of the body fluids to agglutinate rabbit corpuscles in those fluids which had the power to agglutinate them previous to the injection; there is no development, except in a few cases, of agglutinins in the cerebrospinal fluid and the aqueous humor. Altho Table 2 does not show this point, a careful comparison of this table with the succeeding ones

will show that the relative concentration of the agglutinins, in the body fluids, remain the same during the process of immunization.

That there is, however, a marked increase in the amboceptor content of the body fluids, normally containing them, during the process of immunization is shown by Table 3.

TABLE 3.

LYTIC AND AGGLUTINATING POWER ON RABBIT CORPUSCLES OF THE BODY FLUIDS OF DOG IMMUNIZED WITH RABBIT BLOOD AS AFFECTED BY COMPLEMENT (0.2 C.C. FRESH GUINEA-PIG SERUM).
(Intraperitoneal injections of rabbit blood as follows: December 1, 5 c.c.; December 5, 7½ c.c.; December 10, 10 c.c.; December 15, 15 c.c.; December 21, 16 c.c.; January 16, 20 c.c. Fluids collected February 8.)

Dilution	Serum				Neck Lymph				Thoracic Lymph			
	No Complement		Complement		No Complement		Complement		No Complement		Complement	
	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.
1:6	100	—	100	—	60	+	70	+	100	—	100	—
1:12	100	—	100	—	10	+	60	+	70	—	100	—
1:24	90	+	100	—	0	+	40	+	20	+	60	+
1:48	10	+	100	—	0	+	20	+	0	+	50	+
1:96	0	+	50	+	0	0	0	0	0	+	30	+
1:384	0	+	30	+	0	0	0	0	0	0	0	0
1:1,536	0	0	10	0	0	0	0	0	0	0	0	0
1:6,144	0	0	0	0	0	0	0	0	0	0	0	0

Dilution	Pericardial Fluid				Cerebrospinal Fluid				Aqueous Humor			
	No Complement		Complement		No Complement		Complement		No Complement		Complement	
	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.
1:6	0	+	20	+	0	0	0	0	0	0	0	0
1:12	0	+	10	+	0	0	0	0	0	0	0	0
1:24	0	+	0	+	0	0	0	0	0	0	0	0
1:48	0	+	0	+	0	0	0	0	0	0	0	0
1:96	0	0	0	0	..	..	..	..	..	..	..	..
1:384	..	..	..	..	..	..	..	..	..	..	..	..
1:1,536	..	..	..	..	..	..	..	..	..	..	..	..
1:6,144	..	..	..	..	..	..	..	..	..	..	..	..

This experiment shows very clearly that in the immunized animal the serum, neck lymph, thoracic lymph, and pericardial fluid do not contain complement in sufficient quantity to activate all of the amboceptor present in the fluid, because the addition of guinea-pig complement,* in doses of itself not lytic, is able to produce stronger

* We experienced considerable difficulty in securing a complement which was effective and at the same time did not of itself produce hemolysis. Rabbit serum, guinea-pig serum, and dog serum from which the amboceptor had been separated in the cold were tried. The guinea-pig serum proved the only effective one.

hemolysis where only traces had appeared, and to produce hemolysis in other cases where there were no traces with exactly the same amount of the fluid without complement.

The addition of complement in a non-hemolytic dose is able to cause hemolysis in the pericardial fluid. In 8 of 13 experiments on blood-immune dogs traces of hemolysis occurred in the lowest dilutions of that fluid. In these experiments we could detect no contamination with blood, neither did we note that the fluid was present in excessive amounts. Apparently, then, in dogs immune to a foreign blood, amboceptor is always, and complement usually present in the pericardial fluid. This agrees in part with the findings of Mioni who found amboceptor but no complement for guinea-pig corpuscles in the pericardial fluid of the ox. No amboceptors are found in the cerebrospinal fluid or aqueous humor, for no hemolysis occurs when effective complement is added, at least a complement which proved effective in the case of the other body fluids.

The agglutinins in the various body fluids are seen from Table 5 to run practically the same course as in the normal animal, except that the concentration is highest in the serum, a little lower in the thoracic lymph, still lower in the neck lymph, and lowest, but always present, in the pericardial fluid. Sometimes, as in the normal animal, the concentration of agglutinins in the thoracic lymph is equal to or greater than that of the serum. In the 16 immune animals in which we compared serum and thoracic lymph, in 11 cases the serum was higher in the concentration of the agglutinins than the thoracic lymph, in two cases the thoracic lymph was higher than the serum, and in three cases the two fluids showed equal concentration. The fact that the thoracic lymph may contain these antibodies in equal or even considerably higher concentration than the corresponding serum may be of significance as bearing upon the source of these substances.

In 15 experiments with cerebrospinal fluid from immune dogs, a positive agglutination was secured in two. In 16 experiments with the aqueous humor, positive results were secured in five. Thus it will be seen that agglutinins may be found in the cerebrospinal fluid and the aqueous humor, but their presence is the exception and not the rule.

A point of considerable interest is the determination whether the

fluids of an animal immune to one kind of blood show an increased hemolytic and agglutinating power toward the corpuscles of another species. An experiment of this kind is shown in Table 4.

TABLE 4.

LYTIC AND AGGLUTINATING ACTION ON HORSE AND RABBIT CORPUSCLES OF BODY FLUIDS OF A DOG INJECTED WITH HORSE SERUM.

(100 c.c. of serum injected intraperitoneally January 16; fluids removed January 29.)

Dilution	Serum				Neck Lymph				Thoracic Lymph			
	Horse Corpuscles		Rabbit Corpuscles		Horse Corpuscles		Rabbit Corpuscles		Horse Corpuscles		Rabbit Corpuscles	
	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.
1:6	100	–	100	–	100	–	50	+	100	–	100	–
1:12	100	–	100	–	90	+	10	+	100	–	40	+
1:24	100	–	30	+	60	+	0	+	50	+	0	+
1:48	.70	+	10	+	10	+	0	0	20	+	0	0
1:96	30	+	0	+	0	+	0	0	0	+	0	0
1:384	0	+	0	0	0	+	0	0	0	+	0	0
1:1,536	0	+	0	0	0	0	0	0	0	+	0	0
1:6,144	0	+	0	0	0	0	0	0	0	0	0	0

Dilution	Pericardial Fluid				Cerebrospinal Fluid				Aqueous Humor			
	Horse Corpuscles		Rabbit Corpuscles		Horse Corpuscles		Rabbit Corpuscles		Horse Corpuscles		Rabbit Corpuscles	
	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.
1:6	80	+	0	+	0	0	0	0	0	0	0	0
1:12	30	+	0	0	0	0	0	0	0	0	0	0
1:24	0	+	0	0	0	0	0	0	0	0	0	0
1:48	0	+	0	0	sp	–	0	0	0	0	0	0
1:96	0	+	0	0	0	0	0	0	0	0	0	0
1:384	0	0	0	0	..	..	..	..	..	..	..	..
1:1,536	..	..	..	..	..	..	..	..	..	..	..	..
1:16,144	..	..	..	..	..	..	..	..	..	..	..	..

Horse corpuscle control=0. Rabbit corpuscle control=0.

The animal tested here, while showing a marked immunity to horse corpuscles, does not show immunity toward rabbit corpuscles much higher than the normal. Indeed, in the 10 normal animals studied, in two cases the lysins in the neck lymph were as concentrated as here; and in five cases the lysins in the thoracic lymph were as concentrated as here. Therefore, the lysins for rabbit corpuscles are apparently little more concentrated as may be seen by comparing Table 4 with Table 1. The agglutinins are somewhat higher than normal. Apparently the immunity is not entirely specific.

Muir and Browning[32] have advanced some evidence that a com-

plement-like body plays a rôle in agglutination. They used ox corpuscles, rabbit serum immune to ox blood, and guinea-pig serum as complement. They do not make the claim that the complement and the agglutinin are identical, merely that this form of complement behaves like hemolytic complement, is thermostable, and acts only when suitable amboceptor is present. They are not sure whether or not this is the same complement concerned in hemolysis. In the course of our experiments we found some evidence which points in the opposite direction; viz., addition of complement or at least of rabbit serum, as in one experiment, inhibits the agglutination of rabbit corpuscles by the fluids of an immune dog.

TABLE 5.

THE INHIBITION OF THE AGGLUTINATION OF RABBIT CORPUSCLES BY THE BODY FLUIDS OF AN IMMUNE DOG BY THE ADDITION OF RABBIT SERUM.

(150 c.c. rabbit blood intraperitoneally, February 16. Fluids drawn February 26. 0.1 c.c. rabbit serum as complement.)

Dilution	Serum				Neck Lymph				Thoracic Lymph			
	No Complement		Complement		No Complement		Complement		No Complement		Complement	
	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.
1:6	100	–	100	–	100	–	100	–	100	–	100	–
1:12	100	–	100	–	10	+	30	+	100	–	100	–
1:24	80	+	100	–	0	+	0	+	40	+	100	–
1:48	10	+	70	+	0	+	0	+	0	+	10	+
1:96	0	+	10	+	0	+	0	+	0	+	0	+
1:384	0	+	0	+	0	+	0	0	0	+	0	+
1:1,536	0	+	0	0	0	+	0	0	0	+	0	0
1:6,144	0	+	0	0	0	0	0	0	0	+	0	0

Dilution	Pericardial Fluid				Cerebrospinal Fluid				Aqueous Humor			
	No Complement		Complement		No Complement		Complement		No Complement		Complement	
	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.	Lysis	Aggl.
1:6	20	+	20	+	0	+	0	0	sp	–	0	0
1:12	0	+	10	+	0	+	0	0	0	0	0	0
1:24	0	+	0	+	0	+	0	0	0	0	0	0
1:48	sp	–	0	+	0	+	0	0	0	0	0	0
1:96	0	+	0	0	..	..	..	..	..	..	..	..
1:384	sp	–	0	0	..	..	..	..	..	..	..	..
1:1,536	..	..	..	..	..	..	..	..	..	..	..	..
1:6,144	..	..	..	..	..	..	..	..	..	..	..	..

Corpuscle control=0. Complement control=0.

This experiment shows that the addition of rabbit serum as complement instead of favoring agglutination as in the case observed by

Muir and Browning actually inhibited it in every series in the experiment by at least two dilutions. In this case we have a cerebrospinal fluid and an aqueous humor which contained agglutinins for rabbit corpuscles but in these fluids the addition of rabbit serum completely inhibited the action. We would not give the impression that this occurred normally in our work, or even frequently, for in the nine cases where complement was used with body fluids of immune dogs, this phenomenon was noted in only this one experiment. It appeared only once in the fluids of normal dogs, and in that case the action was less marked.

Conclusions.—1. In the blood of dogs immunized with alien blood hemolysins are found in the serum, thoracic lymph, and neck lymph, and usually in the pericardial fluid. They are not found in the cerebrospinal fluid or aqueous humor. The comparative concentration is the same as in the normal animal.

2. The addition of guinea-pig serum as complement in non-hemolytic doses increases greatly the hemolytic power of the serum, neck lymph, thoracic lymph, and pericardial fluid; therefore, in the course of immunization the amboceptors are increased in the fluids, while complement is not. Cerebrospinal fluid and aqueous humor do not become hemolytic on the addition of complement; therefore, they do not contain amboceptors.

3. In the immunized dog the agglutinins are more concentrated than in the same fluids of the normal animal. The usual order of descending concentration is: serum, thoracic lymph, neck lymph, pericardial fluid; but the order may be thoracic lymph, serum, neck lymph, pericardial fluid. Cerebrospinal fluid and aqueous humor may have agglutinins present but usually do not. If agglutinins are present in these two fluids, the concentration is about equal and lower than the pericardial fluid.

4. Immunization of a dog to horse serum increases the hemolytic power of the body fluids for horse corpuscles, but little if at all for rabbit corpuscle. The hemagglutinins are increased to a slight extent.

5. Occasionally the addition of rabbit serum will inhibit the agglutination of washed rabbit corpuscles by the fluids of a dog immune to rabbit blood.

PROTEIN PRECIPITINS.

Since most investigators who have worked with precipitins are agreed as to the delicacy and specificity of the reaction, we chose them as one of the antibodies best suited for study in our work on the body fluids of normal and immune animals. In several cases these were the same animals used in the work on hemolysins and hemagglutinins.

Our method was the same as is usually employed, namely, a dilution method. Doses of the immune fluid varying between 0.2 c.c. and 0.01 c.c. were placed in a series of test tubes and made up to 2 c.c. with sterile 0.9 per cent NaCl solution. To these tubes were then added 0.15 c.c. of the same serum as used for immunization. Control experiments were made in case of each of the fluids, and of the serum, to eliminate any possibilities of a sediment from the protein solutions confusing the results. The tubes were incubated for two hours at 37° C., and then left in the ice-box 12 to 20 hours before the final reading was made.

Our results were as follows: In the fluids of three normal dogs tested with the fresh serum of the rabbit not a trace of precipitate appeared in any tube.

In experiments with the fluids of seven dogs immune to rabbit blood, three gave positive and four negative results. One dog gave a precipitate only in the first dilution of the serum (1:10); the other mixtures and the control mixtures remained perfectly clear. This animal had been immunized by repeated injections of rabbit blood intraperitoneally, receiving in all 57 c.c. between November 14 and December 5, 1908.

A dog which 10 days earlier had received an intraperitoneal injection of 150 c.c. of rabbit blood gave a positive reaction in both serum and thoracic lymph. In this case the precipitation occurred in a much higher dilution than in the former, tho in the latter, neither the neck lymph, pericardial fluid, cerebrospinal fluid, nor the aqueous humor nor any of the controls showed any precipitate.

A dog immunized by the intraperitoneal injection of 80 c.c. of rabbit blood gave the best results of all, the serum and thoracic lymph giving precipitation in dilutions of 1:40 and the neck lymph

in 1:20; the pericardial and cerebrospinal fluids and the aqueous humor, however, gave no reaction in 1:10.

Of the five attempts to produce precipitins in dogs by immunization with horse serum, four were entirely negative, and even in the one positive result only the serum, thoracic lymph, and the neck lymph contained precipitin. The precipitation occurred in a higher dilution in the thoracic lymph than in the serum, but only in the lower dilutions of the neck lymph, which was considerably weaker than the serum.

Our data are not uniform enough nor extensive enough to warrant us in drawing conclusions. It is apparent that dogs develop precipitins with extreme difficulty, and that a good method of immunization is by single, large, intraperitoneal injections. The results that we have seem to show that the precipitins follow closely the hemagglutinins and the hemolysins in their distribution in the various body fluids of immune animals, altho we have not as yet been able to demonstrate any in the pericardial fluid.

BACTERIAL AGGLUTININS.

We also made a study of the concentration of the agglutinins for the typhoid bacillus in the various body fluids of normal and immunized cats and dogs, and intend later to extend the work to cover the bacteriolysins.

Nuttall,[34] employing both the hanging drop method and the plate method, found bacteriolysins for the anthrax bacillus in the aqueous humor and the pleuritic exudate of dogs and rabbits. Prudden[38] found bacteriolysins in the amniotic, hydrocele, and acites fluids. Meltzer and Norris[28] found the thoracic lymph nearly as bacteriolytic as the serum for the typhoid bacillus. Widal[43] found agglutinins for the typhoid bacillus active in a dilution of 1:60 in the pericardial fluid, while the serum of the same patient was active at 1:350. Edema fluid was found strongly agglutinating also, but the result with cerebrospinal fluid was negative. Pick[37] found that cerebrospinal fluid agglutinated at a dilution of 1:1 and 1:2. Kohler[20] found agglutinins in the cerebrospinal fluid in one case in ten examined of non-typhoid patients, and but three times out of 19 cases of typhoid fever, and these in dilutions of 1:1, 1:5, and 1:10. Braude and Carlson[7] made a study of the concentration of the agglutinins for the typhoid bacillus in the body fluids of normal and immunized cats and dogs. Inasmuch as they used for comparison the action of the fluids of the same animal upon the same bacterial suspension, their results give more nearly the true conditions of the body fluids in an animal; they avoided the error which a comparison of the body fluids of different animals is sure to introduce on account of the wide individual variations. The hanging drop method which they employed, however, is not considered as accurate as the pre-

cipitation method which we used. They found bacterial precipitins in the cerebrospinal fluid of immunized dogs and cats.

The fluids used in these experiments were collected as described at the beginning of this paper. The test tubes were the same as those used in the hemolytic work. The bacteria were secured from 20–24-hour slant agar cultures, made up with sterile 0.9 per cent NaCl solution to a distinctly cloudy suspension, and then filtered through filter paper to remove all of the clumps. A series of tubes was arranged for each fluid, then after the proper amount of fluid had been measured into each, the bacterial suspension was added. The dilutions used were 1:10, 1:50, 1:100, 1:500, 1:2,000, 1:6,000. Our lowest dilution was, perhaps, too high to detect the traces of agglutinins reported by Pick and Kohler. The tubes were all incubated together at 37° C. for two hours and then kept in the ice-box for from 12–20 hours before the final observations.

In our study of agglutinins for the typhoid bacillus we used normal cats and dogs, animals actively immunized by the repeated injection of typhoid cultures, and animals rendered passively immune by the withdrawal of large quantities of blood from the normal animal, and the subsequent injection of an equal amount of warm, defibrinated blood from an actively immunized animal of the same kind.

A. Normal animals.—We studied first the concentration of agglutinins in the body fluids of normal cats and found agglutinins for the typhoid bacillus active in a dilution from 1:10 in the serum and the thoracic lymph; the neck lymph and the pericardial fluid usually contain them in the same concentration but the cerebrospinal fluid and the aqueous humor do not contain them in such dilution.

DOGS.—A study of the concentration of the typhoid agglutinins in normal dogs gave the results shown in the following table:

TABLE 6.

THE COMPARATIVE AGGLUTINATING POWER ON THE TYPHOID BACILLUS OF THE BODY FLUIDS OF A NORMAL DOG.

Dilution	Serum	Neck Lymph	Thoracic Lymph	Pericardial Fluid	Cerebro-spinal Fluid	Aqueous Humor
1:10	++	++	++	0	0	0
1:50	++	0	0	0	0	0
1:100	+	0	0	0	0	0
1:500	0	0	0	0	0	0

This experiment is one of four performed on normal animals, and gives a fair idea of the concentration of the agglutinins in the body fluids. There are, of course, variations between animals even in the same species. In all of our experiments the concentration of agglutinins was highest in the serum, less in the thoracic and the neck lymph, and the pericardial fluid contained agglutinins in only one case. The cerebrospinal fluid and the aqueous humor did not contain any in any case.

B. Actively immunized animals.—We made a study of the fluids of five immunized cats. Three had received repeated subcutaneous injections of typhoid bacilli. For the first few injections killed cultures, but later live cultures were used. The doses injected were increased gradually and careful record of the weight and general condition kept, to guard against pushing the process too rapidly. The remaining two animals received a single large injection of six living 24-hour cultures. The results given in Table 7 may be taken as fairly typical.

TABLE 7.

THE COMPARATIVE AGGLUTINATING POWER ON THE TYPHOID BACILLUS OF THE BODY FLUIDS OF AN IMMUNIZED CAT.

(Immunized by repeated subcutaneous injections. Fluids collected five days after the last injection.)

Dilution	Serum	Neck Lymph	Thoracic Lymph	Pericardial Fluid	Cerebro-spinal Fluid	Aqueous Humor
1:10	++	++	++	+	?	?
1:50	++	++	++	0	0	0
1:100	++	++	++	0	0	0
1:500	++	0	+	0	0	0
1:2,000	+	0	0	0	0	0
1:6,000	+	0	0	0	0	0

Control = 0.

The other experiments showed some variations. In two cases in five the agglutinins were as concentrated in the thoracic lymph as in the serum, in the remaining three of five the serum was by far the more concentrated of the two. In one case the neck lymph showed the same concentration of agglutinins as the thoracic lymph, but both were considerably lower than the serum; in the four remaining cases the concentration in the neck lymph was lower than in the thoracic lymph. Agglutinins were found in four of five cases in the pericardial fluid, but in no case in a dilution higher than 1:10. Agglutination in the fifth case was questioned. The cerebrospinal fluid

was negative in all dilutions used in four of five cases, and agglutination in the fifth case at 1:10 was questioned. The aqueous humor gave negative results in three of five cases, and the agglutination was questioned in the remaining two at a dilution of 1:10.

The development of agglutinins for the typhoid bacillus in dogs runs a course which is strictly comparable to that in the cats under similar circumstances.

TABLE 8.

THE COMPARATIVE AGGLUTINATING POWER FOR THE TYPHOID BACILLUS OF THE BODY FLUIDS OF AN IMMUNE DOG.

(Immunized by repeated subcutaneous injections.)

Dilution	Serum	Neck Lymph	Thoracic Lymph	Pericardial Fluid	Cerebro-spinal Fluid	Aqueous Humor	Control
1:10	++	++	++	++	0	0	0
1:50	++	++	++	++	0	0	..
1:100	++	++	++	+	0	0	..
1:500	++	+	++	0	..	..	..
1:2,000	+	0	+	0	..	..	..
1:6,000	0	0	0	0	..	..	..

From Table 8 it is evident that the concentration of the agglutinins in the body fluids of immune dogs runs a course parallel to that in the immune cats. The concentration in the neck lymph and the pericardial fluid is considerably lower than that in the thoracic lymph and serum, and of the two the concentration in the neck lymph is the higher. In our seven experiments on typhoid immune dogs, thoracic lymph and serum showed the same concentration of bacterial agglutinins in four cases; in the remaining three cases the concentration is greater in the serum. The pericardial fluid contained agglutinins in six of seven cases. The highest dilution at which agglutination occurred was 1:100 (three cases). In no case were agglutinins found in the cerebrospinal fluid in the dilutions used. Traces of agglutinins were observed in three of six cases in the aqueous humor in a dilution of 1:10.

We considered it of interest to determine whether immunity to rabbit blood affected in any way the agglutinins for the typhoid bacillus in the body fluids of a dog. With this in mind we tested the usual six fluids of one of our immune dogs. An increased agglutinating power could be expected, if, in terms of Ehrlich's hypothesis, there were any cell receptors common both to blood cells and the typhoid bacilli.

TABLE 9.

THE COMPARATIVE AGGLUTINATING POWER ON THE TYPHOID BACILLUS OF THE BODY FLUIDS OF A DOG IMMUNIZED WITH RABBIT BLOOD.

(Intraperitoneal injections as follows: October 10, 10 c.c.; October 19, 8 c.c.; October 24, 8 c.c.; October 29, 9 c.c.; November 3, 10 c.c.; November 9, 10 c.c.; November 14, 10 c.c.; fluids drawn November 24.)

Dilution	Serum	Neck Lymph	Thoracic Lymph	Pericardial Fluid	Cerebro-spinal Fluid	Aqueous Humor
1:10	++	+	+	0	0	0
1:50	+	0	+	0	0	0
1:100	+	0	0	0	0	0
1:500	0	0	0	0	0	0

A comparison of this table with Table 7 shows that the agglutinins for the typhoid bacillus were little if any higher than those of the normal animal, altho this was one of our most highly immunized animals and agglutinated rabbit corpuscles strongly in a dilution of 1:1,536 in the serum and in a dilution of 1:384 in the neck and the thoracic lymph. The relative concentration in the body fluids is strictly comparable to those in the normal animal cited. We tested also the blood serum of two other dogs immune to rabbit blood with exactly similar results.

C. Passive immunity.—Evidence that antibodies of various kinds are able to pass through membranes is not lacking in the literature. Ehrlich[10] found that the young from a mouse immune to abrin, ricin, or robin possess an immunity to these poisons which persists for two months. Ascoli[1] found that the antibodies of the new born child come from the maternal circulation and are not formed in the fetus itself. This being true, the antibodies of the fetus have penetrated the walls of at least a double membrane. Merkle[29] found that the same was true for rabbits, since the young born of a mother immune to human blood contain antibodies for human blood, altho they suckle a normal mother from the very first. Lüdke[23] confirmed the work of Merkle. Ricketts[39] has shown that the young born to a guinea-pig mother immune to Rocky Mountain spotted fever possess an immunity to that disease, altho they suckled a normal mother. DeBlasi[8] has shown that if a cat is immunized *post partum* to B. dysenteriae the young develop an immunity from the milk. This is even a more striking example than the others of the ability of antibodies to penetrate membranes, for in this case the antibodies after reaching the alimentary canal of the young animal must penetrate

the intestinal mucosa, and later the capillary or lymphatic wall, depending upon whether the path of absorption was the blood or the lymph.

It was hoped that a careful study of the passage of these antibodies from the blood into the various body fluids in a passively immune animal would throw some light upon the problem of lymph formation. We have been disappointed thus far, for our results have not been decisive enough to warrant any general conclusions.

Passive immunity was established by the withdrawal under light ether anesthesia in most cases of a large amount of blood from a normal animal, and the subsequent injection of an equal amount of warm, defibrinated blood from an actively immune animal of the same kind.

CATS.—To show the concentration of agglutinins in the body fluids of a passively immune cat we give Table 10 as typical of our results. It will also show the concentration of agglutinins of the actively immune cat from which the blood was taken.

TABLE 10.

THE COMPARATIVE AGGLUTINATING POWER ON THE TYPHOID BACILLUS OF THE BODY FLUIDS OF CATS ACTIVELY AND PASSIVELY IMMUNE TO THE TYPHOID BACILLUS.

(Cat 11, immunized June 29 by the injection subcutaneously of six live 24-hour slant agar cultures of the typhoid bacillus, operated July 9. Cat 12, passively immunized by the withdrawal on July 9 of 100 c.c. of blood and the injection of 100 c.c. of blood from Cat 11. Operated July 10.)

Dilution	Serum			Neck Lymph		Thoracic Lymph		Pericardial Fluid		Cerebrospinal Fluid		Aqueous Humor	
	11	12*	12	11	12	11	12	11	12	11	12	11	12
1:10	++	+	++	++	++	++	++	+	0	0	0	0	0
1:50	++	+	+	++	+	++	+	+	0	0	0	0	0
1:100	++	0	+	+	0	+	+	0	0	0	0	0	0
1:500	+	0	+	+	0	+	0	0	0	0	0	0	0
1:2,000	+	0	0	+	0	+	0	0	0	0	0	0	0
1:6,000	0	0	0	0	0	0	0	0	0	0	0	0	0

* Normal serum before injection of immune serum 11.

From Table 10 it is evident that it is possible to increase the agglutinins not only in the serum but in both of the lymphs by a process of passive immunization. The relative concentration is maintained in the fluids which we find in the actively immunized animals of the same grade of immunity. In no case in the passively immunized animal did the pericardial fluid show any increase over the normal animal. The cerebrospinal fluid and the aqueous humor showed no

increase, and, indeed, one would not expect that they would, since in the actively immune animal the presence of antibodies is the exception and not the rule. The results here are typical of all of our results. In four passively immune cats this same increase in the concentration of the agglutinins in serum, neck lymph, and the thoracic lymph was noted. In two of the four cases the concentration in the neck lymph and the thoracic lymph was equal, in the remaining two of the four experiments the concentration in the thoracic lymph was greater than that in the neck lymph. There appears on the whole to be a tendency for the concentration of the agglutinins in the thoracic and the neck lymph in the passively immune animals to run more nearly parallel than in the actively immunized animals.

DOGS.—Passive immunity produced in dogs in the manner described yields exactly similar results; namely, the concentration in agglutinins of the serum, neck lymph, and thoracic lymph can be greatly increased. The concentration of the agglutinins in the pericardial fluid, cerebrospinal fluid, and the aqueous humor is no higher than in the normal animal.

In one of three passively immunized dogs the concentration in the neck lymph and thoracic lymph was equal; in the other two the thoracic lymph was much higher. The fact that 50 per cent of our passively immunized cats and 33 per cent of the passively immune dogs showed an equal concentration of the agglutinins in the thoracic and the neck lymph may be significant, for the percentage of such findings in the actively immune animals is 14 per cent for the dogs and 16 per cent for the cats; but much more extensive experimentation is necessary before any reliance can be placed on these results.

In order to determine the relative rapidity with which these antibodies pass from the blood into the lymph the following experiment was made. A dog was anesthetized and rendered passively immune by the withdrawal of 300 c.c. of blood from the femoral artery and the injection of 300 c.c. of blood and serum from a typhoid immune dog. Small samples of blood were drawn at stated intervals, as were also samples of neck lymph. Thoracic lymph, pericardial fluid, cerebrospinal fluid, and aqueous humor were collected at the end of the experiment. All of the fluids except the thoracic lymph

which was tinged with blood were in good condition and were tested in the usual manner. The results are shown in Table 11.

TABLE 11.

THE RATE OF PASSAGE OF ANTIBODIES FROM THE BLOOD INTO THE BODY FLUIDS IN PASSIVELY IMMUNE ANIMALS.

Fluid	Highest Dilution Showing Agglutination
Normal serum	1:100
Immune serum injected into passively immune dog	1:2,000
Equal parts of normal and immune sera	1:2,000
Serum 25 min. after transfusion	1:500
" 1 hr. 25 min. after transfusion	1:500
" 2 " " " " "	1:500
" 3 " " " " "	1:500
" 4 " " " " "	1:500
Neck lymph 3 hr. " "	1:50
" " 4 " 30 min. after transfusion	1:50
Thoracic lymph 4 hr. 30 min. after transfusion	1:100
Pericardial fluid 4 " 30 " " "	0
Cerebrospinal fluid	0
Aqueous humor	0

It may be noted that the results of this experiment are practically identical with those secured from our 24-hour experiments on the passively immunized dogs. The concentration of the agglutinins of the serum remain practically the same during the $4\frac{1}{2}$ hours of this experiment. The neck lymph had the same concentration of agglutinins three hours after the transfusion that it had at the close of the experiment. The thoracic lymph in this case showed considerably higher than the neck lymph, part of which may have been due to the serum in the lymph, which came either through the increased permeability of the capillary walls due to the action of the anesthetic or to trauma, or to the natural anastomoses between the lymphatics and the blood vessels of the splanchnic area.

The above experiment seems to indicate that the passage of such substances as bacterial agglutinins from the blood to the lymphs is a relatively rapid process, for the concentration of these bodies was the same in the body fluids in $4\frac{1}{2}$ hours as in 24 hours after passive immunization.

From the results obtained the following conclusions seem justified:

1. Agglutinins for the typhoid bacillus are found in the serum and thoracic lymph of normal cats in approximately equal amounts.

They may or may not be present in the neck lymph and the pericardial fluid. They are not found in the cerebrospinal fluid nor the aqueous humor.

2. Agglutinins for the typhoid bacillus are found in the serum, neck lymph, and the thoracic lymph in normal dogs in relatively higher concentrations than in the same fluids in normal cats. They are most concentrated in the serum, are in nearly equal concentration in the two lymphs. They may or may not be found in the pericardial fluid. They are found neither in the cerebrospinal fluid nor the aqueous humor.

3. Agglutinins for typhoid bacilli are found in actively immune cats in the serum, thoracic lymph, neck lymph, and pericardial fluid in decreasing concentration in the order mentioned. If found in the cerebrospinal fluid and aqueous humor there are only traces.

4. Agglutinins for typhoid bacilli are found in actively immune dogs in the serum, thoracic lymph, neck lymph, and pericardial fluid, usually in decreasing concentration in the order named. Serum and thoracic lymph may show an equal concentration. Cerebrospinal fluid shows no agglutinins in a dilution of 1:10. Aqueous humor may or may not show agglutinins in a dilution of 1:10.

5. Immunization of a dog to rabbit blood does not increase the concentration of agglutinins for typhoid bacilli in the body fluids above the normal.

6. In the passively immunized animal the agglutinins for typhoid bacilli pass readily from the blood stream into the lymphs, usually in greater concentration into the thoracic lymph than into the neck lymph, but the concentration may be equal in the two, showing the permeability of the two systems to be the same. They do not pass into the pericardial fluid, cerebrospinal fluid, nor the aqueous humor.

7. The passage of the agglutinins from the serum to the lymphs in the passively immunized animal is a relatively rapid process, and no difference is shown between the concentration at the end of 4½ hours and 24 hours.

OPSONINS.

We made a quantitative study of the bacterial opsonins and the hemopsonins in the body fluids of normal and immunized dogs.

Since the discovery of the opsonins by Wright and Douglas[44] there has been a great deal of work done with these bodies, and as a result there has arisen an extensive and conflicting literature. It is beyond the scope of this paper to undertake a general discussion of this subject. We will confine ourselves, therefore, to those results which most nearly relate to our problem. So far as we have been able to determine, there has been up to this time no study of the relative concentration of the opsonins in the different body fluids of the same animal.

Wright and Reid[45] found that exudates may contain little or no opsonin. Opie[35] found that the exudates following the injection of bacteria or turpentine were practically opsonin free if unmixt with blood. Böhme[6] reports 15 cases of pleural and peritoneal exudates showing a concentration of the opsonins ranging from less than half to even a greater concentration than that of the serum. He also examined the edema fluid in seven persons. In two of these cases there was very little opsonin while the remaining five showed a considerable amount, but in no case was it equal to that of the serum. This same investigator examined the cerebrospinal fluid in 16 cases and obtained opsonic indices varying from 8 per cent to 76 per cent. He notes that these fluids were free from blood. Böhme tries to correlate the concentration of the opsonin in these cases with the amount of the protein in the fluid.

Levaditi and Inman[26] and others have shown that while the aqueous humor normally contains little or no opsonin, yet that secured after a previous withdrawal contains the opsonin.

Methods.—The method of securing the fluids was the same as that described in the foregoing.

The leukocytes were from the pleural exudate of young, healthy dogs which 24 hours previously had been given intraplural injection of a suspension of aleuronaut in sterile 0.9 per cent NaCl solution. The exudate was secured after bleeding the animal to death, and was drawn into a warm solution of 1 per cent sodium citrate in 0.9 per cent NaCl. The leukocytes were centrifugated out, and washed a second time in warm, sterile salt solution.

The bacterial emulsion was obtained by suspending in 0.9 per cent NaCl solution 24-hour slant agar cultures of Staph. aureus. This emulsion was filtered through absorbent cotton in order to remove the clumps and was then made up to the desired opalescence. In all of the experiments a fairly rich suspension of the bacteria was used, but no attempt was made to standardize this emulsion, and comparison of individual experiments, except where the same suspension of leukocytes and bacteria were used, would not be warranted.

The technic was essentially that described by Walker with slight modification. This method in our hands gave the most satisfactory results. Dilutions of the various body fluids were used in the same way as the whole fluid. The usual time for incubation was 20 minutes. Care was taken to make the smear from the incubated mixture as soon after the period of incubation as possible, so that the time factor was as nearly equal as it could be kept. We here incur a slight source of error in the difference in the age of the material, for Beattie[5] has shown that the older citrated leukocytes are up to 24 hours, the greater the phagocytosis, but the maximum difference in time between the first and last smear was never greater than one hour. Great care was taken to keep the leukocytic suspension of the same consistency throughout the experiment. The leukocytes were used as fresh as possible, usually in about two hours or less after removal from the animal's body. The number of leukocytes varied from

60 to 100 for each dilution, but the number was kept constant for each experiment. The stains used were carbol-thionin and Giemsa's blood stain, but the same stain was used throughout the same series of fluids.

Inasmuch as there has been so much criticism of the opsonic methods we made the following tests of the accuracy of our observations. Using the same fluids, leukocytes, and bacterial suspension, duplicate tests were made. The slides were labeled in such a way that the person making the count had no way of knowing what the slide contained, thus eliminating the personal equation. The result of this test was that while most of the duplicates agreed fairly closely there were cases that varied as much as 25 per cent, but the majority of the counts showed a much closer agreement.

Bacteriopsonins.—We give the results of an experiment which we consider as typical so far as concerns opsonin for Staph. aureus in normal dogs.

TABLE 12.

COMPARATIVE STUDY OF THE OPSONIN FOR STAPH. AUREUS IN THE BODY FLUIDS OF A NORMAL DOG.

Dilution	Serum	Neck Lymph	Thoracic Lymph	Pericardial Fluid	Cerebro-spinal Fluid	Aqueous Humor
Whole	3.40	3.50	4.17	0.81	1.01	1.08
1:10	0.30	0.16	0.58	0.32	....	....

Control = 0.36.

It will be noted that in this experiment the opsonin had practically disappeared in the dilution of 1:10 in all of the fluids. In the undiluted fluids the concentration of the opsonin in the serum, neck, and thoracic lymph was nearly the same, but there was a slight excess in favor of the thoracic lymph. The pericardial fluid, cerebrospinal fluid, and the aqueous humor contained opsonin in a very much lower concentration.

In four of nine normal animals the concentration of the opsonin in the serum was considerably higher than in the thoracic and the neck lymph. In three of nine cases the concentration was practically equal in the serum and the two lymphs. In five of eight experiments the amount of opsonin in the neck lymph was considerably less than in the serum and thoracic lymph. It is thus evident, that, as far as these three fluids are concerned, the concentration is greatest, on the average, in the serum, least in the neck lymph, while the thoracic lymph occupies an intermediate position.

Opsonin was found in the cerebrospinal fluid of four of seven dogs, but in every case it was in decidedly smaller amounts than in the serum and lymphs of the same animal. In the other three animals there was no opsonin in this fluid.

The aqueous humor contained opsonin in five of eight cases, but in only one of these did the concentration approach that of the serum of the same animal. The remaining four showed very much less phagocytosis, while three experiments gave negative results.

Opsonin was found in the pericardial fluid in two of seven dogs in considerable amounts, in one it was practically equal to the neck lymph of the same animal. In one of the remaining animals there was a trace of opsonin. The other four animals contained no opsonin in this fluid.

It is difficult to increase to any marked extent the concentration of the opsonin for Staph. aureus in a dog by immunization.

We submit below two experiments in detail which we consider quite typical of our results.

TABLE 13.

COMPARATIVE OPSONIC POWER FOR STAPH. AUREUS OF THE BODY FLUIDS OF IMMUNIZED DOGS.

(Both of these dogs received subcutaneously six slant agar cultures of Staph. aureus suspended in 0.9 per cent NaCl solution. Ten days later the fluids were all secured in apparently perfect condition. Fluids kept over night in ice-box. Bacteria from a 24-hour slant agar growth. Incubation 20 minutes. Carbol-thionin. Same bacterial suspension and leukocytes in both experiments.)

	Dilution	Serum	Neck Lymph	Thoracic Lymph	Pericardial Fluid	Cerebrospinal Fluid	Aqueous Humor
Dog I	Whole	9.75	4.51	5.95	3.30	1.85	0.78
	1:10	4.51	1.75	3.51	1.33	0.88	0.50
	1:50	1.75	0.86	1.01	0.22	0.28	0.28
Dog II	Whole	10.07	6.67	8.13	4.18	0.92	1.33
	1:10	4.21	1.50	2.88	1.47	0.22	0.30
	1:50	0.90	0.38	0.52	0.17	0.22	0.35

Control for both = 0.22.

It will be noted that the results of these experiments are comparable with those with respect to the other antibodies studied. In the two experiments cited above the concentration of the opsonin is considerably higher in the serum than in the other fluids. Of the two lymphs there is a slight excess in that from the thoracic duct over that from the cervical lymphatics. In both of these experiments while there is considerable opsonin in the pericardial fluid yet it is less than in the serum and the lymphs. In both of these experiments the cerebrospinal fluid and the aqueous humor contained opsonin, but in very much smaller amounts than in the corresponding serum and lymphs.

Conclusions.—There is a considerable amount of opsonin for

Staph. aureus in the body fluids of normal dogs. The amount found in immune dogs is quite comparable with this, as regards both the amount and the relative distribution.

Opsonin may be present in considerable concentration in all of the body fluids studied by us, but in the serum, thoracic, and neck lymphs they are always found in greater concentration than in the other fluids studied. Of these fluids the serum usually contains the greatest amount, and the thoracic lymph usually more than the corresponding neck lymph. The pericardial fluid, cerebrospinal fluid, and the aqueous humor may or may not contain opsonin, but rarely in amounts comparable to those of the serum and lymphs.

HEMOPSONINS.

It has long been noted that red corpuscles were taken up by phagocytes under certain conditions but it was not until opsonins were discovered by Wright and Douglas that the rôle of the serum in phagocytosis was understood. Neufeld and Töpfer[33] showed that there was something termed by them "hemotropic substance" in the serum of a rabbit immune to goat blood, which caused the phagocytosis of goat erythrocytes by guinea-pig leukocytes *in vitro*. Barrat[3] found the same antibody in the serum of doves immune to hen blood. He proved these bodies thermostable. Hektoen[6] was the first to point out the similarity of these substances to bacterial opsonins and suggested the name "hemopsonins." Neufeld and Töpfer, Barrat, Keith, and Hektoen all cite evidence to show that these substances are distinct from the amboceptors of the hemolysins. The most extensive recent work on the hemopsonins is by Hektoen.[7] He shows that normal serum may contain hemopsonins for heterologous or even homologous erythrocytes; that immune hemopsonins are highly resistant to heat; and are in part specific and in part non-specific.

Methods.—The body fluids of the dogs were secured as described earlier in this paper, and inactivated by heat at 55° C. for 30 minutes. Rat corpuscles were used throughout the work. They were made up to 5 per cent suspension after careful washing in 0.9 per cent NaCl solution. The technic followed was to measure into each of a series of small test tubes quantities of fluids ranging between 0.2 c.c. and 0.002 c.c. and then adding enough salt solution to make 0.2 c.c. To this was then added 0.4 c.c. of a mixture of equal quantities of rat corpuscles and leukocytic suspension. In this way dilutions varying between 1:3 and 1:300 were secured. The tubes were incubated in a shaker for one hour. Then the contents were mixt thoroughly, smears made, and fixt in absolute alcohol for one hour, and then stained with Giemsa's stain for 30–60 minutes. The percentage was figured from the number of the leukocytes actively phagocytic. No attempt was made to count the number of erythrocytes engulfed per leukocyte. In every case 500 leukocytes were counted. Our figures will represent, then, the percentage of leukocytes phagocytic, and shows only the activity of thermostable hemopsonins.

Early in the work an experiment was performed to test the accuracy of the method. Two complete sets of tests were made with the fluids of a normal dog, smears were

made, fixt, and stained in the ordinary way. Labels were then attached to the slides and one of us numbered the slides in a haphazard manner and kept a record of the numbering used. The slides were all mixt together and then counted by the other. In this way the subjective factor would not enter at all. The results were highly satisfactory, for by counting 500 leukocytes and estimating the percentage from that number, the corresponding slides agreed almost exactly. In one case there was a difference amounting to over 2 per cent.

NORMAL DOGS.—The first point was to determine the concentration of the hemopsonins in the body fluids of normal animals. As a typical experiment we cite Table 14.

TABLE 14.

COMPARATIVE HEMOPSONIC POWER FOR RAT CORPUSCLES OF THE BODY FLUIDS OF A NORMAL DOG.

Dilution	Serum	Neck Lymph	Thoracic Lymph	Pericardial Fluid	Cerebro-spinal Fluid	Aqueous Humor
1:3	3.20	1.20	0.60	0.00	0.40	0.20
1:6	0.20	0.00	0.20	0.00	0.00	0.00
1:12	0.20	0.00	0.00	0.00	0.00	0.00

Control=0.

This table shows that the concentration of hemopsonins in the various body fluids runs practically parallel with that of the other antibodies. It is highest in the serum. The two lymphs are lower but practically equal. There are also traces in the other fluids in this case. In another normal animal the same conditions were found.

ACTIVELY IMMUNIZED DOGS.—In establishing immunity for the work on hemopsonins we followed a uniform technic. Each animal received intravenously 0.5 c.c. of a 5 per cent solution of washed rat corpuscles per kilo of body weight. The animal was operated the 10th day after the injection. We cite the experiment in Table 15 as typical.

TABLE 15.

COMPARATIVE HEMOPSONIC POWER FOR RAT CORPUSCLES OF THE BODY FLUIDS OF AN IMMUNE DOG.

Dilution	Serum (Normal)	Serum	Neck Lymph	Thoracic Lymph	Pericardial Fluid	Cerebro-spinal Fluid	Aqueous Humor
1:3	24.4	45.0	43.4	39.4	25.8	10.8	4.8
1:6	4.2	38.4	28.0	28.2	15.0	2.6	1.0
1:12	1.8	24.6	5.0	14.6	7.0	1.2	0.0
1:24	0.4	5.8	3.0	2.2	2.0	0.2	0.0
1:60	0.0	2.6	2.0	0.0	0.0	0.0	0.0
1:120	...	1.0	0.8	0.0	0.0	0.0	0.0
1:300	...	0.2	0.0	0.0	...	...	...

Table 15 shows that a considerable degree of immunity was reached, the immune serum producing a much higher degree of

phagocytosis than the normal. The concentration was highest in the serum; the two lymphs are practically parallel with a slight balance in favor of the neck lymph, because it not only produces a higher percentage of phagocytosis in the lowest dilution, but also causes phagocytosis in a much higher dilution. The pericardial fluid shows a somewhat lower concentration than the lymphs; the cerebrospinal fluid and the aqueous humor contain the opsonin in lower concentration than the pericardial fluid.

In six experiments with immune dogs the concentration of hemopsonins ran the course shown in this experiment. We find some variations in the cerebrospinal fluid and the aqueous humor. In three out of five cases where the cerebrospinal fluid and aqueous humor were tested the concentration of the hemopsonins was higher in the former than in the latter. The fourth case is the one cited in Table 15, and in the fifth case neither showed hemopsonins. In one of six cases the hemopsonins of the pericardial fluid and aqueous humor were practically equal in concentration.

PASSIVELY IMMUNE DOGS.—Passive immunity was produced in the same way described under bacterial agglutinins. We cite here as an example the results obtained in the case of a dog immunized by injecting intravenously 300 c.c. of the blood of an immune dog after first removing 260 c.c. by bleeding.

TABLE 16.

COMPARATIVE HEMOPSONIC POWER FOR RAT CORPUSCLES OF THE BODY FLUIDS OF A DOG PASSIVELY IMMUNIZED TO RAT BLOOD.

Dilution	Serum (Normal)	Serum	Neck Lymph	Thoracic Lymph	Pericardial Fluid	Cerebrospinal Fluid	Aqueous Humor
1:3	26.0	36.4	15.6	32.0	3.6	0.8	0.4
1:6	16.0	27.2	7.8	12.0	1.0	0.0	0.2
1:12	4.6	13.6	1.6	2.6	0.0	0.0	0.0
1:24	0.4	2.6	0.0	1.6	0.0	...	...
1:60	0.0	1.8	0.0	0.4	...	...	...
1:120	...	0.6	...	0.0	...	...	...
1:300	...	...	...	...	...	...	...

The results in our experiment in passive immunity show first, an increase in the concentration of the hemopsonins in the serum, and second, a marked increase in the concentration of the antibodies in the thoracic lymph, bringing it up to a concentration equal to that of the serum, while the concentration of the hemopsonins in the neck

lymph remains low and from a comparison with other animals we would say that the injection of the highly immune serum had had no effect upon the concentration of the hemopsonins in the neck lymph. The same is true of the pericardial fluid, the cerebrospinal fluid, and the aqueous humor, where the concentration is no higher than in the normal dog. It must be remembered that this dog showed a relatively high hemopsonic power before immunization by injection of the immune blood. We have other experiments which show the same phenomenon.

From these experiments we draw the following conclusions:

1. Hemopsonins are found in the body fluids of normal dogs. The concentration is highest in the serum, lower in the thoracic and neck lymph, which run almost parallel, and are found in the other body fluids only in traces.

2. By a process of immunization the hemopsonins of the body fluids can all be increased. The order of descending concentration is the serum, neck lymph, thoracic lymph, pericardial fluid, aqueous humor, and the cerebrospinal fluid. Sometimes the arrangement in the scale of the last two is reversed.

3. Hemopsonins can pass from the blood into the thoracic lymph, but apparently they do not pass into the lymph of the head region. The concentration of the hemopsonins in the pericardial fluid, cerebrospinal fluid, and aqueous humor is not increased in 24 hours over the normal by passive immunization as in our experiments.

GENERAL CONCLUSIONS.

In the normal animal the concentration of hemolysins, hemagglutinins, bacterial agglutinins, bacterial opsonins, and hemopsonins decrease in the body fluids in the following order: serum, thoracic lymph, neck lymph. Traces of hemagglutinins, bacterial agglutinins, and opsonins are found in the pericardial fluid. Traces of opsonins are found in the cerebrospinal fluid and aqueous humor. No precipitins for rabbit serum were found in any of the body fluids.

Immunization increases the concentration of the antibodies named above in the fluids in which they are found in the normal animal. Hemolysins are sometimes found in the pericardial fluid, and in a few cases traces of bacterial agglutinins in the cerebrospinal fluid and

aqueous humor. Protein precipitins are found in the serum, thoracic lymph, and neck lymph, the concentration being lower in the last than in the first two.

The immunity is specific in the other body fluids as in the serum.

The introduction of an immune blood into a normal animal increases the concentration of the bacterial agglutinins in both the lymphs above the normal. The introduction of an immune blood does not increase the concentration of hemopsonins in the neck lymph, altho the concentration in the thoracic lymph is markedly increased. The concentration of bacterial agglutinins and hemopsonins in the cerebrospinal fluid is not modified by passive immunization.

The passage of antibodies from blood to the lymph is a relatively rapid process.

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A STUDY OF COMPLEMENT FIXATION IN GONORRHEAL INFECTIONS.*

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IN the year 1901 Bordet and Gengou[1] first demonstrated the method of "Complement-deviation." Afterward, in 1906, Wassermann and Bruck[2] modified Bordet's original method to what is now called "Komplementablenkungsmethode."[3] Since this method was introduced several varieties of bacteria and also protozoa have been studied by it. The study of syphilis especially by this method has been reported from every part of the world by countless numbers of authors. That it is one of the most remarkable experiments of recent times is shown by the fact that now we cannot discuss syphilis without knowing this method. The question, How does the gonococcus react with complement-binding method? is still unsettled. One year after Wassermann's publication Müller and Oppenheim[4] first studied the serum of a gonorrheal patient with this method. With serum from a patient suffering from gonorrheal arthritis, with gonococcus as antigen (NaCl emulsion of 48-hour culture on blood-agar) and with fresh guinea-pig serum as complement, then adding the beef red corpuscles and a hemolytic serum, they found that the complement was anchored by the patient's serum, hemolysis not occurring. On the contrary, the serum of a patient suffering from non-gonorrheal diseases did not anchor the complement and lysis of the erythrocytes took place. From this fact it is clear that gonorrheal serum contained a specific antibody for gonococcus. Shortly afterward Bruck[5] studied the sera of six cases of gonorrheal salpingitis, and two cases of chronic gonorrheal urethritis and obtained positive results in two of six cases of the former and in one out of the two latter. In 1907 Meakins[6] obtained two positive reactions in three cases of gonorrheal arthritis and positive results from the serum of patients suffering from pro-

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[1] *Ann. de l'Inst. Pasteur*, 1901, 15, p. 290.

[2] *Deut. med. Wchnschr.*, 1906, 32, p. 745.

[3] The term "Komplementablenkung" is frequently called in English Complement-binding, Fixation of complement, Deviation of complement, or Wassermann's reaction.

[4] *Wien. klin. Wchnschr.*, 1906, 19, p. 894.

[5] *Deut. med. Wchnschr.*, 1906, 70, p. 36.

[6] *Johns Hopkins Hosp. Bull.*, 1907, 18, p. 255.

statitis and chronic urethritis. In the same year Vannod[1] studied the serum of a rabbit immunized with the gonococcus. The antigen he used was aqueous extract of gonococcus on ascitic agar prepared by shaking for 48 hours; as complement he used guinea-pig fresh serum and added sheep erythrocytes with a specific hemolytic serum. The results showed that all immune sera anchored the complement strongly, even with the minimum doses of 0.001 c.c. of serum. In the control, normal rabbit serum never anchored the complement, the result being complete hemolysis. Serum of the rabbit immunized with meningococcus also gave complete hemolysis. The same year Wollstein[2] reported the results obtained with four cases of immune sera, using immunized rabbit serum, fresh guinea-pig serum as complement, and chicken erythrocytes with specific hemolytic serum. All immune sera gave positive reactions, no hemolysis occurring. Micrococcus catarrhalis and streptococcus as antigens gave negative results, complete hemolysis occurring; but when the antigen was meningococcus the antigonococcus serum anchored the complement and hemolysis did not occur as with gonococcic antigen; and using antimeningococcus rabbit serum with gonococcic antigen, the results also showed anchoring of the complement. Consequently one cannot differentiate between gonococcus and meningococcus by this method. Teague and Torrey[3] experimented with antigonococcic rabbit serum. The antigen was an aqueous extract of a 24-hour culture of gonococcus on Thalmann's medium. The results showed that all antigonococcal sera anchored the complement strongly, but the results were different from those of the previous workers in this, that the immune serum obtained with gonococcus A reacted strongly only with gonococcus A as antigen (homogeneous) and gave no reaction with gonococcus G or H (heterogeneous); and G- or H-serum reacted strongly only with G- or H-antigen (homogeneous) but not with A-antigen (heterogeneous). From these results they concluded that gonococci might be divided in several types as urged by Torrey[4] in previous contributions on the agglutination and precipitation of gonococcus. This is a very interesting subject for further study. I have also observed recently the same phenomenon with anti-

[1] *Centralbl. f. Bakt.*, Abt. I, Orig., 1907, 44, p. 10.

[2] *Jour. Exper. Med.*, 1907, 9, p. 588.

[3] *Jour. Med. Res.*, 1907, 17, p. 223.

[4] *Ibid.*, 1907, 16, p. 329.

gonococcic sera while working on complement-fixation. The results obtained are shown in the following experiments:

Human serum—

1. Normal serum; 2. Gonorrheal serum; 3. Serum with gonorrheal history; 4. Serum from patients with other diseases.

Rabbit serum—

1. Normal serum; 2. Immunized gonococcic serum; 3. Immunized meningococcic serum; 4. Torrey's gonococcic serum.

Other normal animal sera—

1. Horse serum; 2. Ox serum; 3. Goat serum.

Relation to agglutination.

Conclusions.

Method.—For the experiments five different agents are required; the procedure is as follows:

Amboceptor: 24–48-hour culture of gonococcus on blood-agar (horse blood) emulsified in normal salt solution, and this living emulsion placed in a shaking apparatus for 48 hours, then injected intraperitoneally into a rabbit at intervals of a week to 10 days with increasing doses. About 12–15 days after last injection (8 to 10 times) the rabbit is bled and tested for the experiments.

Antigen: a slant culture of 24–48 hours on blood-agar emulsified in 5 c.c. of sterile distilled water and heated at 60° C. for ½ hour, then placed in a shaking apparatus for 48 hours and centrifugalized. As a result of this preparation the fluid was not, however, completely cleared. The extract was kept in the ice-room and it was never used when more than three days old (just five days after culture). For controls B. coli, meningococcus, and Staph. aureus emulsion were made under the same conditions. For the experiments the gonococcus as antigen had been isolated from the same sources as given in a previous paper[1] by me.

Complement: Normal guinea-pig freshly bled and the serum placed in the ice-room for 16 to 24 hours. Rabbit serum as complement is frequently very variable in power, guinea-pig serum as complement has almost the same power, and I never failed to get complete hemolysis with it. Rabbit complement sometimes showed no hemolysis, unless it was used in large doses, when it caused complete hemolysis, showing that rabbit's serum used as complement is inconstant. My dose of guinea-pig's complement is 0.05 c.c.

Hemolytic sera: Freshly defibrinated goat's blood corpuscles injected intraperitoneally into a healthy rabbit at intervals with increasing doses as 2, 5, 10, and 20 c.c. About 10 days after last injection the serum was tested by experiments as to its hemolytic power for a solution of 5 per cent erythrocytes, from undiluted serum to 0.001–0.0025 c.c. To the hemolytic serum 0.5 per cent carbolic acid was finally added and the whole kept in the ice-room until required for use.

Erythrocytes: Goat's blood corpuscles from freshly defribinated blood, the washed erythrocytes made into a 5 per cent suspension by adding 0.9 per cent salt solution.

Small test tubes were used for experiments. First the antigen (0.1 c.c.), amboceptor (certain doses), and complement (0.05 c.c.) were well mixt in the tubes and placed in incubator at 37° C. for one hour. During this period the tubes were shaken 3 to 4 times. Next the hemolytic sera and erythrocytes were added and the tubes

[1] *Jour. Med. Res.*, 1909, 20, p. 365.

placed in the incubator at 37° C. for 2 hours, shaken 4 or 5 times at intervals. I used certain strengths of hemolytic serum added to 5 per cent erythrocytes; for instance, if the hemolytic title was 0.001 c.c. for 1 c.c. of 5 per cent suspension, 0.1 c.c. of this hemolytic serum was added directly to 100.0 c.c. of 5 per cent suspension, and 1 c.c. of this mixture added to the previous agents. After 2 hours in the incubator, the tubes were kept in ice-room for 24 hours and the results observed. The following table shows details of the technic.

TABLE 1.

	Antigen	Amboceptor			Complement 2×	0.9% NaCl Sol.		5% Erythrocytes with Certain Hemolytic Sera		Results
1	0.1	0.1		1.0	0.05 (0.1)	0	37° C. for 1 hour	1.0	37° C. for 2 hours	after 24 hours in ice-room
2	"	0.05	10×	0.5	"	0.5		"		
3	"	0.025		0.25	"	0.75		"		
4	"	0.01		1.0	"	0		"		
5	"	0.005	100×	0.5	"	0.5		"		
6	"	0.0025		0.25	"	0.75		"		
7	"	0.001	1000×	1.0	"	0		"		
Control I	0	0			0.05	1.0		"		
Control II	0	0.1			0	0		"		
Control III	0.1	0			0.05	1.0		"		
Control IV	0	0.1			0.05	0		"		
Control V	0	0			0	0		"		

NOTE.—Results were indicated as follows: — = no hemolysis, ± = trace, + = hemolysis, ++ = strong hemolysis, +++ = complete hemolysis, ? = not tested.

HUMAN SERUM.

Normal serum.—Before experimenting with gonorrheal serum I examined normal human serum for its reaction. Healthy human serum, with absolutely no history of any gonorrheal infection, was taken from a blister and inactivated at 55° C. for one hour, then tested, the results being as follows:

TABLE 2.

O. K., aged 25, never affected by gonorrhea, at present in healthy condition. His serum did not fix complement.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	+++	+++	+++	+++	—	—	+++	+++	—	+++
0.05	+++	+++	+++	+++	±	±	+++	+++	+	+++
0.025	+++	+++	+++	+++	+	+	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	—	—	—	—	—	—	—	—	—	—
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	—	—	+++	+++	±	+++
Control V	—	—	—	—	—	—	—	—	—	—

As the table shows, complete hemolysis occurred with the gonococcus strains A, B, C, D, G, H, and B. coli, as no complement was fixt, but with gonococcus E and F, and meningococcus no hemolysis occurred in doses of 0.1 c.c. This may be supposed however to be a normal condition like the normal agglutination property of the serum, because 0.1 c.c. of serum is a comparatively large amount. Therefore I think this is a normal condition, not a specific reaction.

TABLE 3.

S. A., aged 27, never affected by gonorrhea, at present suffering from dysentery (bacillary). Serum taken three weeks after the onset of the dysentery.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.05	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control V	–	–	–	–	–	–	–	–	–	–

This serum did not fix the complement for any of the antigens.

Following sera were taken from patients who may or may not have had gonorrhea previously, but at the time were healthy. The answers about a gonorrheal history could not be relied upon; therefore, it is only so-called healthy serum, but whether affected previously or not by gonorrhea could not be known for certain.

TABLE 4.

T. S., aged 30, at present in a healthy condition, denied having ever been affected by gonorrhea.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	H	F	G	H		
0.1	–	–	–	–	–	–	–	–	+	++
0.05	–	–	–	–	–	–	–	–	++	+++
0.025	–	–	–	+	–	++	±	–	+++	+++
0.01	–	+	±	+	–	++	++	+	+++	+++
0.005	+	++	+++	+++	±	+++	++	++	+++	+++
0.0025	++-	+++	+++	+++	++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control V	–	–	–	–	–	–	–	–	–	–

This serum caused no hemolysis because the complement was fixt, showing that T. S. serum might have contained gonococcic amboceptor. I again asked him for a gonorrheal history, but the answer was the same as before, that he never had gonorrhea. Then I asked his friend secretly, who was living in the same room with him. The friend said that T. S. had been suffering from gonorrheal urethritis for the past four weeks. The friend's answer explained clearly the positive reaction as shown by result.

TABLE 5.

S. T., aged 18, present condition healthy, said he never had gonorrheal infection.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	++	+++	−	+++	+	?	++	−	++	+++
0.05	+++	+++	++	+++	++	?	+++	+	+++	+++
0.025	+++	+++	+++	+++	+++	?	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	?	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	?	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	?	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	?	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	?	+++	+++	+++	+++
Control II	−	−	−	−	−	?	−	−	−	−
Control III	+++	+++	+++	+++	+++	?	+++	+++	+++	+++
Control IV	+++	+++	−	+++	+++	?	+++	−	+++	+++
Control V	−	−	−	−	−	?	−	−	−	−

The result shows that the serum had no gonococcic amboceptor. The subject may not have had gonorrheal infection. For this experiment the antigen of gonococcus F was not prepared because, in shaking, the tube was broken by accident.

Serum of gonorrheal patients.

TABLE 6.

R. W., aged 39, first attacked by gonorrhea 20 years ago; since that time he has had a recurrence of the same infection five times. At present his physician diagnosed him as suffering from chronic gonorrheal urethritis.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	++	+++	++	++	+++	+	+	+++	+++	+++
0.05	+++	+++	+++	+++	+++	+++	+	+++	+++	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	−	−	−	−	−	−	−	−	−	−
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+++	+	+	+++	+++	+++
Control V	−	−	−	−	−	−	−	−	−	−

The table shows that the serum has no amboceptor to any other gonococcus strains, than gonococcus E which was isolated from this patient. This homogeneous extract showed complete hemolysis (fixt con plement).

TABLE 7.

Y. M., aged 30, diagnosed as chronic gonorrheal urethritis and cystitis.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	–	–	–	–	–	–	–	–	–	++
0.05	–	–	–	–	+	–	–	–	+	+++
0.025	+	–	±	–	++	–	+	–	++	+++
0.01	+++	++	++	–	+++	–	++	+++	+++	+++
0.005	+++	+++	+++	+	+++	+	+++	+++	+++	+++
0.0025	+++	+++	+++	++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control V	–	–	–	–	–	–	–	–	–	–

Gonococcus F was isolated from this patient. All strains show positive reactions, especially F (homogeneous) and D which reacted in the same manner very strongly. From this it may be assumed that strains F and D belong to the same group.

TABLE 8.

C. H., aged 25, attacked by gonorrheal infection three years ago; since that time the disease has persisted as chronic gonorrheal urethritis, cystitis, and epididymitis.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	–	–	–	–	+	–	–	–	–	?
0.05	–	–	–	–	+	–	–	–	+	?
0.025	–	–	–	+	+	±	–	–	+++	?
0.01	–	–	–	+	++	++	+	–	+++	?
0.005	–	–	+	++	+++	+++	+++	–	+++	?
0.0025	–	–	++	+++	+++	+++	+++	–	+++	?
0.001	++	+	+++	+++	+++	+++	+++	+	+++	?
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	?
Control II	–	–	–	–	–	–	–	–	–	?
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	?
Control IV	+++	+++	+++	+++	+++	+++	+++	+++	+++	?
Control V	–	–	–	–	–	–	–	–	–	?

Gonococcus H was isolated from this patient. Reaction was positive for A, B, C, D, E, F, G, and H (homogeneous). Gonococcus A and B reacted in equal strength to the serum, showing H, A, and B to be of the same group.

TABLE 9.

K. U., aged 35, diagnosed as secondary stage of syphilis and chronic gonorrheal endometritis.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	–	–	–	–	–	–	–	–	+	++
0.05	–	–	–	–	–	–	–	–	+	++
0.025	–	–	–	+	–	±	–	–	+++	+++
0.01	–	–	+	++	–	+	–	–	+++	+++
0.005	–	–	+++	+++	–	+++	±	–	+++	+++
0.0025	+	–	+++	+++	++	+++	++	±	+++	+++
0.001	++	+++	+++	+++	+++	+++	+++	++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control V	–	–	–	–	–	–	–	–	–	–

The gonococcus could not be isolated from this patient, so this serum could not be tested with homogeneous strain as antigen. However, all strains showed positive reactions, especially A, B, C, E, G, and H, which reacted somewhat similarly; therefore it may be assumed that if a gonococcus was isolated from this patient, that gonococcus might have reacted the same as strains A, B, C, E, G, and H and belong to the same group (see Table 19, gncc. imm. serum).

TABLE 10.

T. K., aged 22, acute gonorrheal urethritis, two weeks after infection.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	+++	±	+++	++	+++	+++	+	+	+++	+++
0.01	+++	++	+++	++	+++	+++	+++	++	+++	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	–	+++	+++	+++	+++	+	+	+++	+++
Control V	–	–	–	–	–	–	–	–	–	–

Gonococcus A was isolated from this patient, but the homogeneous extract as the antigen and other heterogeneous antigens did not fix the complement; complete hemolysis occurred. Gonococcus B showed slight hemolysis in 0.1 c.c. but this is a normal reaction as previously mentioned.

TABLE 11.

H. S., aged 20, acute gonorrheal urethritis, five days after infection.

Serum	Gonococcus Strains A	B	C	D	E	F	G	H	Meningococcus	B. coli
0.1	+++	+++	+++	+++	+++	+	+++	+++	+++	+++
0.05	+++	+++	+++	+++	+++	++	+++	+++	+++	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+++	+	+++	+++	+++	+++
Control V	–	–	–	–	–	–	–	–	–	–

Gonococcus C was isolated from this patient, but the results were all negative.

Concerning the last two tables we note that acute gonorrheal sera do not fix the complement to the antigens.

Serum from patients with a history of gonorrhea but no disease at present.

TABLE 12.

K. K., aged 30, first infection five years ago. Affected several times since, but present condition healthy.

Serum	Gonococcus Strains A	B	C	D	E	F	G	H	Meningococcus	B. coli
0.1	–	±	–	–	–	–	–	+++	++	±
0.05	–	+++	±	–	+	–	–	+++	+++	+++
0.025	+++	+++	+++	++	++	+++	–	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	–	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	±	±	++	+	–	+++	+++	+++	+++	+
Control V	–	–	–	–	–	–	–	–	–	–

The results show that gonococcus G fixt the complement to this serum. It may be supposed that if the gonococcus had been isolated from this patient it might be of the same group as the G strain.

TABLE 13.

H. T., aged 25, affected by acute gonorrheal urethritis three years before; since that time he never has been affected again and at present in a healthy condition.

Serum	Gonococcus Strains								Men-ingo-coc-cus	B. coli
	A	B	C	D	E	F	G	H		
0.1	+++	+++	+++	+++	+	+++	+++	++	++	+
0.05	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	—	—	—	—	—	—	—	—	—	—
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control V	—	—	—	—	—	—	—	—	—	—

This shows entirely negative resul s.

Serum from patients with other diseases.

TABLE 14.

S. S., aged 25, serum taken 10 days after diagnosis of typhoid fever. No history of any gonorrheal infection.

Serum	Gonococcus Strains								Men-ingo-coc-cus	B. coli
	A	B	C	D	E	F	G	H		
0.1	+++	+++	?	+++	+++	+++	+++	+++	+++	+++
0.05	+++	+++	?	+++	+++	+++	+++	+++	+++	+++
0.025	+++	+++	?	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	?	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	?	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	?	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	?	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	?	+++	+++	+++	+++	+++	+++	+++
Control II	—	—	?	—	—	—	—	—	—	—
Control III	+++	+++	?	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	?	+++	+++	+++	+++	+++	+++	+++
Control V	—	—	?	—	—	—	—	—	—	—

The result was negative.

TABLE 15.

S. R., aged 24, diagnosed as catarrhal pleurisy; gonorrheal history is unknown; the pleural exudate was examined.

Serum	Gonococcus Strains								Men-ingo-coc-cus	B. coli
	A	B	C	D	E	F	G	H		
0.1	+++	+++	+++	++	+	+++	+++	+++	+++	+++
0.05	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+	+++	+++	+++	+++	+++
Control V	–	–	–	–	–	–	–	–	–	–

The result was negative.

RABBIT SERUM.

Normal rabbit serum.

TABLE 16.

Rabbit I, weight 2,400 gm., healthy.

Serum	Gonococcus Strains								Men-ingo-coc-cus	B. coli
	A	B	C	D	E	F	G	H		
0.1	–	–	+++	+++	+	+++	–	+++	±	±
0.05	±	±	+++	+++	+++	+++	±	+++	+++	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	–	±	++	+++	+	+++	–	+++	+	±
Control V	–	–	–	–	–	–	–	–	–	–

Results are all negative. Some of the experiments, however, showed no hemolysis with 0.1 c.c. of the serum. This may be the limit to normal reaction, not specific.

TABLE 17.
Rabbit III, 2,500 gm., healthy.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	−	+++	−	+++	+	−	+++	+++	+++	+++
0.05	±	+++	±	+++	+++	+	+++	+++	+++	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	−	−	−	−	−	−	−	−	−	−
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	−	+++	±	+++	+	−	+++	+++	+++	+++
Control V	−	−	−	−	−	−	−	−	−	−

The results are similar to normal serum of Rabbit I.

TABLE 18.
Rabbit V, 1,600 gm., healthy, normal.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	±	+	±	+++	+	+++	+++	+++	++	+++
0.05	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	−	−	−	−	−	−	−	−	−	−
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	±	+	±	+++	+	+++	+++	+++	+++	+++
Control V	−	−	−	−	−	−	−	−	−	−

As seen from the three tables above the results are all similar in reaction, and it may be accepted that normal rabbit sera do not fix the complement to gonoccocic antigen, no hemolysis occurring except in 0.1 to 0.05 c.c. which is a normal condition like that of human serum.

Immunized rabbit serum.—Rabbits were highly immunized with eight different strains of gonococcus, consisting of 24- to 48-hour growths from blood-agar (original test tube), emulsified in normal salt solution, and heated at 55° C. for one hour, then injected intravenously with intervals of five days. After third injection, living gonococcic emulsion was injected intraperitoneally at intervals of a week with increasing doses. Altho various strains of gonococcus differ greatly in toxicity, the animals seldom succumb to this treatment. After the injection the animals lost weight markedly and some died

after third or fourth inoculation or even a few days after first injection. It is clear that immunization of animals with gonococcus is not easy, however; usually after the fifth inoculation the animals became thoroughly immunized and began to gain weight. After the rabbit had received 10 inoculations extending over a period of about two months the blood was drawn from the carotid 10 to 15 days after last injection, and the various immunized rabbit sera used for the experiments. The results are as follows:

TABLE 19.

Rabbit Immunized to Gonococcus A.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	–	–	–	–	–	–	–	–	–	+++
0.05	–	–	–	–	–	–	–	–	+	+++
0.025	–	–	–	–	–	–	–	–	++	+++
0.01	–	–	–	+	–	+	–	–	+++	+++
0.005	–	–	–	++	±	++	–	–	+++	+++
0.0025	–	–	±	+++	±	+++	–	+	+++	+++
0.001	+	±	++	+++	+	+++	+	++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+	+++	++	+++	±	+++
Control V	–	–	–	–	–	–	–	–	–	–

As seen from the above results immunized gonococcic serum A reacted strongly to gonococcus A (homogeneous), B, C, E, G, and H. This interested me very much, as by comparing the positive reaction of the gonorrheal patients' sera (see Table 9) to gonococcus strains A, B, and H, it seems to me this shows clearly gonococcus strains A, B, C, E, G, and H belong to the same group.

TABLE 20.

Rabbit Immunized to Gonococcus B.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	–	–	–	–	–	–	–	–	±	+++
0.05	–	–	–	–	–	–	–	–	+++	+++
0.025	–	–	–	–	–	–	–	–	+++	+++
0.01	–	–	–	+	–	±	–	–	+++	+++
0.005	–	–	–	++	–	+	–	–	+++	+++
0.0025	–	–	–	+++	+	++	–	–	+++	+++
0.001	–	–	–	+++	+	++	±	–	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control V	–	–	–	–	–	–	–	–	–	–

The results show that strains A, B, C, E, G, and H are of the same group.

TABLE 21.

RABBIT IMMUNIZED TO GONOCOCCUS C.

SERUM	GONOCOCCUS STRAINS								MEN-INGO-COC-CUS	B. COLI
	A	B	C	D	E	F	G	H		
0.1	—	—	—	—	—	—	—	—	+	+
0.05	—	—	—	—	—	—	—	—	+++	++
0.025	—	—	—	±	—	±	—	—	+++	+++
0.01	—	—	—	++	—	+	—	—	+++	+++
0.005	—	—	—	+++	—	++	—	—	+++	+++
0.0025	±	+	—	+++	±	+++	—	—	+++	+++
0.001	+	+	±	+++	++	+++	+	—	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	—	—	—	—	—	—	—	—	—	—
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+	+++	—	+++	++	+++	++	+	+	+
Control V	—	—	—	—	—	—	—	—	—	—

As seen from the above strains C, and A, B, E, G, H, belong to the same group.

TABLE 22.

RABBIT IMMUNIZED TO GONOCOCCUS D.

SERUM	GONOCOCCUS STRAINS								MEN-INGO-COC-CUS	B. COLI
	A	B	C	D	E	F	G	H		
0.1	—	—	—	—	—	—	—	—	—	±
0.05	—	—	—	—	—	—	—	—	±	+++
0.025	—	+	+	—	+	—	+	+++	+++	+++
0.01	+++	+++	+++	—	+	—	+	+++	+++	+++
0.005	+++	+++	+++	—	+	—	+	+++	+++	+++
0.0025	+++	+++	+++	—	+	±	+++	+++	+++	+++
0.001	+++	+++	+++	—	+	+	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	—	—	—	—	—	—	—	—	—	—
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	—	++	+++	+	+++	+++	+++	+++	+	±
Control V	—	—	—	—	—	—	—	—	—	—

The results show that strains D and F give the same reaction, that is, they belong to the same group. The previous table also shows D and F had the same reaction from gonorrheal patients' sera. There is a decided difference between the D and F, and the A, B, C, E, G, and H strains.

TABLE 23.
RABBIT IMMUNIZED TO GONOCOCCUS E.

SERUM		GONOCOCCUS STRAINS								MEN-INGO-COC-CUS	B. COLI
		A	B	C	D	E	F	G	H		
0.1		–	–	–	–	–	–	–	–	–	–
0.05		–	–	–	–	–	–	–	–	++	++
0.025		–	–	–	–	–	+	–	–	+++	+++
0.01		–	–	–	++	–	++	–	–	+++	+++
0.005		–	–	–	+++	–	+++	–	–	+++	+++
0.0025		–	–	–	+++	±	+++	–	–	+++	+++
0.001		+	±	±	+++	+	+++	–	–	+++	+++
Control	I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	II	–	–	–	–	–	–	–	–	–	–
	III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	IV	+	+	–	–	++	+	–	+++	±	–
	V	–	–	–	–	–	–	–	–	–	–

The results show that gonococcus E serum reacted to A, B, C, G, and H strains, i. e., they all belong to the same group.

TABLE 24.
RABBIT IMMUNIZED TO GONOCOCCUS F.

SERUM		GONOCOCCUS STRAINS								MEN-INGO-COC-CUS	B. COLI
		A	B	C	D	E	F	G	H		
0.1		–	–	–	–	–	–	–	–	+++	+++
0.05		–	–	–	–	–	–	–	–	+++	+++
0.025		–	–	±	–	–	–	–	–	+++	+++
0.01		±	±	+	–	+++	–	+	+	+++	+++
0.005		+++	++	++	–	+++	–	+++	+	+++	+++
0.0025		+++	+++	+++	–	+++	–	+++	+	+++	+++
0.001		+++	+++	+++	+	+++	–	+++	+++	+++	+++
Control	I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	II	–	–	–	–	–	–	–	–	–	–
	III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	IV	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	V	–	–	–	–	–	–	–	–	–	–

This shows that strains D and F clearly belong to the same group.

TABLE 25.
RABBIT IMMUNIZED TO GONOCOCCUS G.

SERUM		GONOCOCCUS STRAINS								MEN-INGO-COC-CUS	B. COLI
		A	B	C	D	E	F	G	H		
0.1		–	–	–	–	–	–	–	–	++	+++
0.05		–	–	–	–	–	–	–	–	+++	+++
0.025		–	–	–	–	–	–	–	–	+++	+++
0.01		–	–	–	++	–	±	–	–	+++	+++
0.005		–	–	–	++	–	+++	–	–	+++	+++
0.0025		–	+	+	+++	–	+++	–	–	+++	+++
0.001		–	+	+	+++	–	+++	–	±	+++	+++
Control	I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	II	–	–	–	–	–	–	–	–	–	–
	III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	IV	+++	+++	+	++	+++	+++	++	+++	++	+++
	V	–	–	–	–	–	–	–	–	–	–

This shows, strain G and strains A, B, C, E, H, are of the same group.

TABLE 26.

RABBIT IMMUNIZED TO GONOCOCCUS H.

SERUM	GONOCOCCUS STRAINS								MENINGOCOCCUS	B. COLI
	A	B	C	D	E	F	G	H		
0.1	–	–	–	–	–	–	–	–	+++	+++
0.05	–	–	–	–	–	–	–	–	+++	+++
0.025	–	–	–	–	–	–	–	–	+++	+++
0.01	–	–	–	++	–	+	–	–	+++	+++
0.005	–	–	–	+++	–	+	–	–	+++	+++
0.0025	–	+	–	+++	+	+++	–	–	+++	+++
0.001	–	+	±	+++	+	+++	–	–	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control V	–	–	–	–	–	–	–	–	–	–

This shows again that H and A, B, C, E, and G, belong to the same group.

Meningococcus immune rabbit serum.

TABLE 27.

RABBIT IMMUNIZED TO MENINGOCOCCUS.

SERUM	GONOCOCCUS STRAINS								MENINGOCOCCUS	B. COLI
	A	B	C	D	E	F	G	H		
0.1	+	+++	–	+++	–	–	+++	+++	–	+++
0.05	+++	+++	+++	+++	+	–	+++	+++	–	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	–	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	–	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	–	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	–	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	–	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+	+++	±	+++	–	–	+++	+++	++	+++
Control V	–	–	–	–	–	–	–	–	–	–

The results show that all gonococcus strains have not reacted positively. Normal reaction, however, was limited to the serum of 0.1 to 0.05 c.c. dilutions. On the other hand meningococcic antigen reacted positively with strong fixation of complement, showing that a certain differentiation between meningococcus and gonococcus may be determined by this method. At present time does not permit me to test this reaction upon several strains of meningococcus; it must be reserved for future observations.

TORREY'S ANTIGONOCOCCIC SERUM.

The Tokio branch office of Parke Davis & Co. supplied me with the sample of Torrey's antigonococcic serum. It may be added that Torrey's serum is prepared by immunizing a goat with several stains of gonococcus. The serum which I used was two months old, counting from the time it was sent out from the Parke Davis laboratory. I have tested this comparatively old Torrey's serum upon the complement-binding reaction, the results being as follows:

TABLE 28.

TORREY'S ANTIGONOCOCCIC SERUM.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	–	–	–	–	–	–	–	–	–	+
0.05	–	–	–	–	–	+	–	–	++	+++
0.025	–	–	–	±	–	+	–	–	+++	+++
0.01	+	–	–	+	–	+	–	–	+++	+++
0.005	++	+	+++	+	+	+++	+	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	–	–	–	–	–	–	–	–	–	–
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	±	+++	+++	+++	++	±	–	+
Control V	–	–	–	–	–	–	–	–	–	–

As seen from the above table the results show the same reactions to all gonococcus strains. This preparation of course is old and contained probably several different strains of gonococcus and it may be has lost its original power of "complement-binding." If the serum was new it might react positively and strongly. In any case I could not find any different strains or what groups were contained in this sample of Torrey's antigonococcic serum from my eight strains of gonococcus.

OTHER NORMAL ANIMAL SERA.

I have examined the sera of the horse, ox, and goat; the results are as follows:

TABLE 29.
NORMAL HORSE SERUM I.

Serum	Gonococcus Strains								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	+++	+++	+++	±	+++	+++	+++	+++	+++	+++
0.05	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	—	—	—	—	—	—	—	—	—	—
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	++	+++	+++	+++	+++	+++	+++
Control V	—	—	—	—	—	—	—	—	—	—

TABLE 30.
NORMAL HORSE SERUM II.

Serum	Gonococcus Strains								Meningococcus
	A	B	C	D	E	F	G	H	
0.1	—*	—*	±	+*	+	—*	—*	±	+
0.05	++	+++	+++	+++	+++	++	+++	++	++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	—	—	—	—	—	—	—	—	—
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	—*	—*	+	++	++	—*	—*	±	+++
Control V	—	—	—	—	—	—	—	—	—

* Normal phenomenon: this is more frequent in horse serum than other serum.

As seen from the tables normal horse serum never fixes the complement to gonococcic antigen.

TABLE 31.
NORMAL OX SERUM I.

Serum	Gonococcus Strain								Meningococcus	B. coli
	A	B	C	D	E	F	G	H		
0.1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.05	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	—	—	—	—	—	—	—	—	—	—
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control V	—	—	—	—	—	—	—	—	—	—

TABLE 32.

NORMAL OX SERUM II.

SERUM	GONOCOCCUS STRAINS A	B	C	D	E	F	G	H	MENINGOCOCCUS	B. COLI
0.1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.05	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control II	—	—	—	—	—	—	—	—	—	—
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control IV	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Control V	—	—	—	—	—	—	—	—	—	—

Normal ox serum does not fix the complement to any gonococcic antigens.

TABLE 33.

NORMAL GOAT SERUM.

SERUM	GONOCOCCUS STRAINS A	B	C	D	E	F	G	H	MENINGOCOCCUS	B. COLI
0.1	+	+++	+	+	+++	+	+++	+	+++	?
0.05	+	+++	+	+++	+++	+++	+++	+++	+++	?
0.025	+++	+++	+++	+++	+++	+++	+++	+++	+++	?
0.01	+++	+++	+++	+++	+++	+++	+++	+++	+++	?
0.005	+++	+++	+++	+++	+++	+++	+++	+++	+++	?
0.0025	+++	+++	+++	+++	+++	+++	+++	+++	+++	?
0.001	+++	+++	+++	+++	+++	+++	+++	+++	+++	?
Control I	+++	+++	+++	+++	+++	+++	+++	+++	+++	?
Control II	—	—	—	—	—	—	—	—	—	?
Control III	+++	+++	+++	+++	+++	+++	+++	+++	+++	?
Control IV	+	+++	+	+	+++	+	+++	++	+++	?
Control V	—	—	—	—	—	—	—	—	—	?

As seen from the above tables sera of normal animals never react positively for any strains of gonococcus.

RELATION TO AGGLUTINATION.

My observation of agglutination, with the same strains of gonococcus as tested with the complement-binding reaction, showed that there is no relation between complement-binding reaction and agglutination. This fact agrees with Bruck's studies. The details of the experiments will be published in the near future.

CONCLUSIONS.

The study of different species of bacteria is an important problem at the present time. Several strains of B. dysenteriae and B. coli and others have been described and are differentiated by their reaction on carbohydrate media, but observations of the gonococcus upon carbohydrate media have not yet shown any different strains. Torrey and Teague have studied the gonococcus in regard to the agglutination, precipitation, and complement-binding reactions and have shown that the strains may be divided into several types. According to their experiments from ten strains three different groups were isolated. Can the gonococcus be proved to have several species by the serum reaction? I have studied this question by the complement-binding method, the results being as above described. Concerning my experiments, eight strains of gonococcus may be separated into two different groups. There is not, however, a distinct difference between them like that of different types of B. dysenteriae as shown on carbohydrate media. It seems that the gonococcus has not many types, but at present our knowledge of the different types of bacteria is still imperfect. I failed to isolate many different groups and my experiments, unlike the results of Torrey, have not shown any distinct differentiation among them. His experiments have shown only positive reactions to homogeneous, but never positive to heterogeneous strains; it is, therefore, difficult to differentiate between various types of gonococcus and other bacteria by this method. But my experiments have shown no distinct difference among the various strains of gonococcus, only a comparative difference, and it is very easy to differentiate between any gonococcus and other bacteria.

The following general conclusions may be drawn from the experiments I have described:

1. Normal human serum has frequently marked hemolysis in 0.05 to 0.1 c.c. of the serum. This is not a specific reaction, but seems to be a normal reaction like normal agglutination (group reaction). In normal serum, however, hemolysis never occurred in dilutions below 0.05 c.c. of the serum.

2. The sera of gonorrheal patients contain specific amboceptors for the complement-binding reaction. In chronic cases the results are usually positive. Of six cases on which I carried out my experi-

ments three chronic cases showed positive reactions and one chronic and two acute cases gave negative reactions.

3. In serum of previously gonorrheal patients, well at the time of the test, one chronic case was positive and one acute case negative.

4. The sera of patients suffering from other diseases, viz., typhoid fever and catarrhal pleurisy, showed negative reaction.

5. Of the three experiments carried out with normal rabbit serum no complement was fixt for any strains of gonococcus. In doses of 0.1 c.c. of the serum, however, hemolysis frequently marked the reaction like the normal reaction of normal human serum.

6. Of eight strains of immunized rabbit serum six gave a strong reaction for six certain gonococcus strains which may belong to the same group, and two sera gave strong reaction for two certain gonococcus strains which may be classified as another group; so this divides the eight strains into two different groups. But this difference is only comparative.

7. As the control antimeningococcic serum gave a positive reaction for meningococcic antigen only, and a negative for gonococcic antigen, this reaction will differentiate between these species. Also the complement-binding reaction upon the different species of gonococcus was stronger than the reaction with meningococcus used as control. It is not, therefore, difficult to differentiate meningococcus from gonococcus by this method.

8. As controls normal sera of other animals, two horses, two oxen, and one goat, were tested. The sera all gave negative reactions and the so-called normal reaction did not occur as with normal human and rabbit sera.

9. There is no relation whatever between the complement-binding reaction and the agglutination reaction.

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VENOM HEMOLYSIS.*

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GENERAL zoölogical interest as to the structure of snake venoms is augmented by the special importance accredited these secretions in the field of pathology, and a basis for this importance exists in the close structural resemblances which the venoms bear to the toxins produced by pathogenic bacteria. Like the bacterial toxins, the venoms are the highly specialized products of living cells; they are non-crystallizable and chemically undefined; they effect, in minute doses, constant and characteristic pathological changes.

The bacterial toxins possess as a prime characteristic the power of stimulating the production of specific antitoxins. The venoms likewise possess this power, and the phenomena occurring in the process of such an immunization are strictly analogous to those occurring in the immunization with bacterial toxins.

Closely related to antitoxin production is the phenomenon of "toxoid" formation. A bacterial toxin may be so modified by destructive agents that, although deprived of its toxicity, it retains the power of effecting antitoxin production. Venoms similarly treated also display the phenomenon of toxoid formation and herein again strikingly agree with a characteristic property of the true toxins.

Extreme complexity of composition so well recognized for the bacterial toxins is also paralleled in the case of the venoms. While

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in the case of the chemically defined poisons, mercuric chloride for example, the multiplicity of intoxication phenomena is explained by referring them to one and the same poison acting on the several different organs, in the case of the bacterial toxins it must now be recognized that the multiplicity of toxic phenomena depends upon the plurality of dissimilar components which go to make up the composite toxin. Thus, following Ehrlich's conception of special chemical affinities between given cell-groups and individual toxin components, it has been possible to resolve tetanus toxin into at least two toxic components—tetanospasmin and tetanolysin; to which Tizzoni would add as a third constituent a substance responsible for the production of cachexia. In much the same manner and to an equal degree, extreme complexity has been demonstrated as a structural characteristic of the venoms. Thus Myers, employing destructive agents, was able to differentiate a hemolytic from the neurotoxic principle in cobra venom, and more recently there has been described a venom constituent acting specifically on the intimal endothelium of blood vessels.

These, and other marked coincidences between the characteristic properties of the venoms and those of the bacterial toxins are of significant import to workers investigating the bacterial toxins, and especially so since the degree of coincidence seems to justify an expectation that findings as to the structure of venoms may in a degree be applicable to an analysis of the bacterial toxins.

Further, certain limiting conditions which attend a study of the bacterial toxins are less restricting in the examination of venoms. The admixture of culture media, so confusing a complication with the bacterial toxins, is not a factor with the venoms. Then too, the instability of many of the bacterial toxins, so militating against accurate quantitative experiments, although not entirely eliminated in the case of the venoms is reduced to a relatively comfortable working minimum. The venoms offer in addition a certain uniformity of product not approximated by the bacterial toxins.

To a degree influenced by the considerations thus outlined, I have during the past five years conducted investigations as to the structure of venoms, the results of which it is the purpose of this paper to present. These investigations deal primarily with the hemolytic

constituent of venoms and more especially with that of the Indian cobra (Naja tripudians).

The experiments were conducted for the most part at the Royal Prussian Institute for Experimental Therapy, and at this point it is a pleasure to express unqualified gratitude for the keen and sympathetic counsel at all times afforded by Professor Ehrlich, the director of that institute. To Dr. Hans Sachs I am indebted for collaboration in certain of the experiments herein referred to and which have been conjointly reported by us in another place. Acknowledgment is also made of generous financial support at various times afforded by the Rockefeller Institute for Medical Research and by the Memorial Institute for Infectious Diseases.

For convenience, the following divisions are observed in the text:

I. Lytic Action of Venoms upon Serum-free Erythrocytes.
II. Intracellular Activation.
III. Lecithin Activation.
IV. Isolation of Cobra Lecithid.
V. Serum Activation.
VI. General Considerations.

I.

LYTIC ACTION OF VENOMS UPON SERUM-FREE ERYTHROCYTES.

The destruction of red blood corpuscles which results in the escape of hemoglobin from the stromata is, for the purpose of this paper, designated as hemolysis, and those components of snake venoms which effect this phenomenon are indicated as venom hemolysins or hemotoxins. In the analysis of the action of these hemolysins, so extensive recourse has been made to quantitative test-tube experiments that a statement of the general methods of procedure in such experiments forms an essential preface to the discussion of the more specific data.

As a criterion by which to grade the hemolytic power of venoms, a constant blood unit was employed as a standard indicator. This unit indicator, representing the amount of blood added to each tube of a series, was arbitrarily fixed as 1 c.c. of a 5 per cent suspension of serum-free erythrocytes in an 0.85 per cent aqueous solution of sodium chloride, prepared as follows:

Freshly defibrinated blood of a normal adult centrifugalized and the supernatant serum (approximately ½ the total volume) withdrawn; the sediment of erythrocytes

suspended in sufficient 0.85 per cent NaCl solution to represent a 1:40 blood dilution (2.5 per cent); this suspension centrifugalized and the sediment resuspended in the same amount of salt solution; this lavage repeated at least twice and the resulting sediment finally suspended in sufficient salt solution to represent a 5 per cent suspension relative to the volume of blood as shed.

The venoms and other reagents tested upon this blood unit indicator were also dissolved or suspended in 0.85 per cent NaCl and were added in graduated amounts to tubes in series.[1] The total content of each tube was brought to an approximate volume equivalent of 1 c.c. by the addition, where necessary, of a supplemental amount of 0.85 per cent NaCl solution; then was added the blood indicator (1.0 c.c.), making the total in each tube approximately 2 c.c. Immediately subsequent to the addition of the blood indicator the tubes were thoroughly shaken and placed at 37° C. At this temperature the reaction was allowed to proceed for two hours, after which the tubes were again shaken and placed at 6° to 8° C. for from 14 to 20 hours. The readings of the grade of hemolysis were made at the close of this period and are known to represent the end phase of each reaction.

Coupled with the special conditions indicated for individual experiments, these general lines of procedure give uniform quantitative results. It must be emphasized, however, that with so delicate an indicator as the cytological one here employed, slight deviations from a stated method may be made to produce results in no way comparable.

The hemolytic action of venoms determined by the methods thus outlined, far from being uniform for all bloods, is found to vary markedly with the species of animal from which the blood is obtained. Thus, according to their susceptibility to cobra venom erythrocytes may be divided, primarily, into two groups, viz.: (1) those which are dissolved by cobra venom alone; (2) those which are dissolved by cobra venom only in conjunction with accessory reagents (complements, etc.).

In illustration of these differences the susceptibility and non-susceptibility of the washed corpuscles of several species of the commoner experimental animals and of man to cobra venom are shown in the following table (Table 1).

From this table it is seen that the erythrocytes of the guinea-pig, dog, man, mouse, frog, rabbit, rat, goose, pig, and horse are dissolved by cobra venom alone, whereas those of the ox, sheep, and goat are not dissolved even by relatively large doses of the venom;

[1] The percentage of concentration indicated for the venoms is relative to native venom desiccated *in vacuo* to constant weight.

in fact, no concentration of the venom, however great, effects a solution of the corpuscles of the latter group.

TABLE 1.

SUSCEPTIBILITY OF ERYTHROCYTES OF DIFFERENT SPECIES TO COBRA VENOM.

AMOUNT OF 1 PER CENT COBRA VENOM C.C.	1 C.C. 5 PER CENT SUSPENSION OF ERYTHROCYTES						
	Frog	Dog	Guinea-Pig	Man	Rat	Swine	Mouse
1.0	complete	complete	complete	complete	complete	....	complete
0.1	"	"	"	"	"	complete	"
0.05	"	"	"	"	"	"	"
0.025	"	"	"	"	"	"	"
0.01	"	"	"	"	almost complete	marked	medium
0.005	"	"	"	"	marked	trace	trace
0.0025	"	"	"	almost complete	faint trace	0	0
0.001	"	marked	slight	medium	0	0	0
0.0005	marked	trace	trace	trace	0	0	0
0.00025	slight	faint trace	faint trace	faint trace	0	0	0
0.0001	0	0	0	0	0	0	0

AMOUNT OF 1 PER CENT COBRA VENOM C.C.	1 C.C. 5 PER CENT SUSPENSION OF ERYTHROCYTES					
	Goose	Rabbit	Horse	Ox	Sheep	Goat
1.0	....	....	....	0	0	0
0.1	complete	complete	complete	0	0	0
0.05	"	almost complete	trace	0	0	0
0.025	almost complete	slight	faint trace	0	0	0
0.01	marked	0	0	0	0	0
0.005	faint trace	0	0	0	0	0
0.0025	0	0	0	0	0	0
0.001	0	0	0	0	0	0
0.0005	0	0	0	0	0	0
0.00025	0	0	0	0	0	0
0.0001	0	0	0	0	0	0

Table 1 further shows that here, as in general with true toxin hemolysins, there exists between the several susceptible species marked differences in the degree of susceptibility. Thus the amount of venom required to dissolve frog's corpuscles is but $\frac{1}{100}$ the amount required for the solution of rabbit or horse corpuscles. Indeed, as might be expected from the behavior of true toxins, a certain difference in degree of susceptibility is not infrequently found between individuals of the same species. Such differences most frequently occur with the rabbit and horse, and the following table indicates the degree of variation shown by the blood of four rabbits reacting with the same specimen of cobra venom (Table 2).

From this table it is seen that between individuals of the same species there may exist a distinct variation in the lytic dose, a difference which in extreme cases is tenfold in extent. On the other hand, of the susceptible species no individual has been found whose erythrocytes were not dissolved by some concentration of cobra venom, and vice versa, no individual of a non-susceptible species has been found whose erythrocytes could be dissolved by any concentration of cobra venom.[1]

TABLE 2.

DIFFERENCES IN SUSCEPTIBILITY TO COBRA VENOM OF ERYTHROCYTES FROM INDIVIDUALS OF SAME SPECIES.

Amount of Cobra Venom (1.0 per cent) c.c.	1 c.c. 5 per cent Rabbit Erythrocytes			
	Rabbit No. I	Rabbit No. II	Rabbit No. III	Rabbit No. IV
1.0	o	....	....	....
0.5	faint trace	....	....	....
0.25	slight	....	....	....
0.1	complete	o	trace	complete
0.075	faint trace	complete	....	"
0.05	o	medium	marked	"
0.025	o	o	complete	"
0.01	o	o	trace	"
0.005	o	o	o	marked
0.0025	o	o	o	trace
0.001	o	o	o	faint trace
0.0005	o	o	o	o

Furthermore, of 22 specimens of cobra venom not one was found which failed to dissolve the erythrocytes of all the species designated as susceptible, or which produced hemolysis of any of the corpuscles designated as non-susceptible. In other words, such variations as occur are quantitative and not qualitative.

The underlying structural differences which determine that the erythrocytes of one species shall be dissolved by cobra venom, whereas those of another species shall not, are discussed in other sections of this paper (II and III). At this point it is only indicated that the fact be established that certain species of corpuscles are dissolved by the venom alone, whereas those of other species are not. This fact is fundamental in that the normal susceptibility of any given species of erythrocytes is the basis of all quantitative experimentation as to activation of venoms by complements, etc., and especial

[1] It is intended here to discuss only the blood of normal adults. For differences between fetal and adult blood see H. Sachs, *Centralbl. f. Bact.*, Abt. 1, 1903, 34, p. 686.

emphasis upon this point is indicated in view of contradictions existing in the literature.

Flexner and Noguchi[1] in an early paper concerning the activation of venoms took the position that no erythrocytes are hemolyzed by venom alone. Working with cobra, water moccasin, copperhead, and rattlesnake venoms, and with dog, rabbit, guinea-pig, sheep, ox, and pig erythrocytes, these authors cited as a result "that in no instance were the washed blood corpuscles hemolyzed by venom." Shortly following the appearance of this statement I was forced to conclude from experiments comparable to those given in Table I that the erythrocytes of many species, including several of those cited by Flexner and Noguchi, are dissolved by cobra venom alone, and so stated.[2] Somewhat later, however, in a second paper Flexner and Noguchi[3] retained their position that washed erythrocytes in general are not susceptible to hemolysis by venoms alone, and emphasized the possibility of an apparent susceptibility due to insufficient lavage in the preparation of the cells. Such hemolysis by cobra venom as could not be prevented by sufficient washing of the cells, these authors referred to as "partial hemolysis after some hours." Finally, Noguchi[4] has come to the view that some kinds of corpuscles are dissolved by venom alone.

In view of these contradictions I can but emphasize the fact that cobra venom constantly produces a hemolysis of many species of erythrocytes, which hemolysis cannot rightly be designated as partial and which is not to be referred to a lack of lavage in the preparation of the cells. Since the early work of Stephens[5] there has been no doubt as to the favorable influence of certain sera upon venom hemolysis and hence as to the necessity of removing the serum before testing the susceptibility of the erythrocytes *per se*. But that a remaining trace of serum is not a factor determining the susceptibility of the erythrocytes tested in the experiments above cited, is evidenced by two facts. The first of these facts is that simple mathematical calcula-

[1] *Jour. Exper. Med.*, 1902, 6, p. 277.

[2] *Berl. klin. Wchnschr.*, 1902, 39, pp. 886, 918.

[3] *Univ. of Penna. Med. Bull.*, 1902, 15, p. 345.

[4] *Jour. Exper. Med.*, 1907, 9, p. 436.

[5] *Jour. of Path. and Bact.*, 1900, 6, p. 273. Also thesis published at the University of Cambridge, November, 1898.

tion shows that a single washing of the erythrocytes in the bulk of salt solution indicated so dilutes the admixed serum that the amount of serum contained in 1 c.c. of a 5 per cent suspension is less than the amount necessary to effect activation even by the most potent serum. As a matter of fact two such washings were invariably made. The second fact which eliminates serum activation as a factor in the case of the washed susceptible corpuscles is that, whereas the serum of certain species produces no activation of cobra venom whatsoever, the corpuscles of the species show a distinct susceptibility. It is evident that here the serum, even were it actually present as a result of insufficient washing, could not be held to account for the susceptibility of the erythrocytes. The blood of the rabbit offers such an example and the following table suffices for explicit data (Table 3).

TABLE 3.

EFFECT OF THE PRESENCE OF RABBIT SERUM UPON THE HEMOLYSIS OF RABBIT ERYTHROCYTES BY COBRA VENOM.

AMOUNT OF COBRA VENOM (1 PER CENT) c.c.	1 C.C. 5 PER CENT RABBIT ERYTHROCYTES +		
	Cobra Venom Alone	Cobra Venom + 0.5 c.c. Rabbit Serum	Cobra Venom + 0.05 c.c. Rabbit Serum
0.1	complete	0	0
0.075	medium	0	0
0.05	faint trace	0	0
0.035	0	0	0

Here it is seen that whereas the washed erythrocytes of the rabbit are dissolved by venom alone, the presence of serum of the same animal not only does not activate the venom but actually inhibits lysis otherwise occurring.

Finally it may be shown that not only is there a susceptibility of erythrocytes to cobra venom in the absence of serum, but that there exists a susceptibility which is not modified by maximum lavage. The following table shows the susceptibility of the same preparation of rabbit corpuscles at various stages of washing (Table 4).

From this table it may be seen that the susceptibility of these cells is not only independent of serum but also uninfluenced by multiple washings. In view of these facts, the constant results cited in Table 1 stand as a contradiction of the position that washed erythrocytes are not dissolved by venom alone.

It might be argued that the power of cobra venom to effect typical hemolysis of washed erythrocytes is the unusual property of certain anomalous specimens of venom, but in view of the fact that all of the 22 specimens which I have tested are constant in producing this effect I should rather conclude that the normal was here represented and that a specimen which lacked such power represented an anomalous or attenuated venom. Nor should the impression be conveyed that the power of hemolyzing washed corpuscles is peculiar to the venom of the cobra. It is rather a power possessed by venoms in general. With a single exception, the venoms of 10 species of snakes tested show a lytic action for the corpuscles of one or more species of blood. For completeness in illustration of this, the following table is included. The figures parenthetically placed in the last column represent the amount of each venom required for the solution of guinea-pigs' blood (Table 5).

TABLE 4.

EFFECT OF REPEATED WASHINGS OF RABBIT ERYTHROCYTES UPON HEMOLYSIS BY COBRA VENOM.

AMOUNT OF COBRA VENOM (1 PER CENT) C.C.	1 C.C. 5 PER CENT RABBIT ERYTHROCYTES				
	Washed 2×	Washed 4×	Washed 6×	Washed 8×	Washed 10×
0.1	complete	complete	complete	complete	complete
0.075	"	"	"	"	"
0.05	marked	marked	marked	marked	marked
0.035	medium	medium	medium	medium	medium
0.025	slight	slight	slight	slight	slight
0.015	trace	trace	trace	trace	trace
0.01	0	0	0	0	0

TABLE 5.

ACTION OF DIFFERENT VENOMS UPON SHEEP, RABBIT, HUMAN, AND GUINEA-PIG ERYTHROCYTES.

VENOM OF	BLOOD OF			
	Sheep	Rabbit	Man	Guinea-Pig
Bothrops lanceolatus	0	0	0	0
Trimeresurus anamallensis	0	0	0	+(1.0 mg.)
Crotalus	0	0	0	+(0.5 mg.)
Trimeresurus riukiuanus	0	0	+	+0.25 mg.)
Ancistrodon piscivorus	0	0	+	+(0.15 mg.)
Bungarus coeruleus	0	0	+	+(0.1 mg.)
Bungarus fasciatus	0	0	+	+(0.1 mg.)
Naja haje	0	0	+	+(0.05 mg.)
Daboia russellii	0	0	+	+(0.035 mg.)
Naja tripudians (Cobra)	0	+	+	+(0.025 mg.)

From this table it is seen that of the 10 venoms tested, nine dissolve guinea-pig corpuscles, seven dissolve the corpuscles of man, and one dissolves rabbit corpuscles. The corpuscles of the sheep are not dissolved by any venom. It is also to be noted that there exists a certain correspondence between the intensity of the lytic action of a given venom with a susceptible blood, and the number of species of blood which that venom dissolves. Thus cobra venom, whose lytic action for guinea-pig corpuscles is shown to be the greatest, also dissolves the greatest number (3) of bloods tested. Whereas Bungarus fasciatus, which requires a dose four times that of cobra venom to dissolve guinea-pig corpuscles, dissolves but two species of blood, and Trimeresurus anamallensis, which requires a dose 40 times that of cobra to dissolve guinea-pig corpuscles, dissolves this most susceptible species only. Bothrops lanceolatus fails to dissolve any of the species tested. Judged, therefore, both from the extent of their reactions with a given susceptible blood and from the number of species with which they react, cobra venom may be cited as the most intense of those venoms tested, and Bothrops venom as the least so.

Summarizing, then, the data as to the action of venoms upon serum-free erythrocytes, it appears that:

1. Venoms vary in hemolytic power according to their species of origin.

2. Venoms in general possess the power of dissolving the erythrocytes of certain species but lack this power for the erythrocytes of certain other species.

3. For cobra venom the susceptible species include guinea-pig, dog, man, mouse, frog, rabbit, rat, goose, pig, and horse, whereas the non-susceptible species include ox, sheep, and goat.

II.

INTRACELLULAR ACTIVATION.

The recognition of two groups of erythrocytes, the susceptible and the non-susceptible, as indicated in the preceding section, suggests an apparent contradiction as to the structure of cobra hemotoxin. The fact that certain erythrocytes are not dissolved by the venom alone, and that these erythrocytes undergo hemolysis upon the addi-

tion of serum, would seem to indicate an amboceptor structure for the hemotoxin. On the other hand, the fact that certain other erythrocytes are dissolved by the venom without the addition of complements, would appear to argue equally well that the hemolysin is a simple toxin complete in itself and not an amboceptor.

In attempting an analysis of this apparent contradiction it might be urged that cobra venom contains two distinct and dissimilar hemotoxins, the one of amboceptor structure acting only in the presence of suitable serum complements, the other a simple toxin requiring no such activation and responsible for the hemolysis of those erythrocytes designated as susceptible. It must then further be assumed that the simple toxin possesses the power of reacting with the corpuscles of certain species only. As a matter of fact, however, the primary assumption of the presence of a simple hemotoxin is not in accord with the experimental data. Thus, as early as 1898 Stephens and Myers[1] showed that whereas certain erythrocytes are dissolved by cobra venom, these same erythrocytes remain undissolved, if the dosage of venom be sufficiently increased. This observation I have been able to extend to washed corpuscles and especially to those of the rabbit (Table 6).

TABLE 6.

NON-LYTIC ACTION OF MAXIMUM DOSES OF COBRA VENOM WITH WASHED ERYTHROCYTES.

AMOUNT OF COBRA VENOM (1 PER CENT)	1 C.C. 5 PER CENT SUSPENSION RABBIT ERYTHROCYTES	
C.C.	Rabbit A	Rabbit B
1.0	0	....
0.5	faint trace	....
0.25	slight	....
0.1	complete	0
0.075	faintest trace	complete
0.05	0	medium
0.025	0	0
0.01	0	0

The above table shows that with washed susceptible corpuscles larger doses of venom may fail to effect hemolysis where smaller doses readily do so, and this occurrence contraindicates the assumption of a simple toxin, inasmuch as with simple toxins an increase in dosage implies an increase in toxic action. The phenomenon cited

[1] *Jour. of Path. and Bact.*, 1898, 5, p. 279.

above suggests rather that the hemolysin here acting is of the nature of a complex toxin, the force of the suggestion being in the fact that the single analogous phenomenon thus far observed occurs with a lysin known to be a complement-amboceptor complex. Neisser and Wechsberg[1] showed in the case of bacteriolytic immune sera that with a fixed amount of complement, a sufficient increase in the amount of amboceptor so deviates the complement as to produce a complete inhibition of the bacteriolysis otherwise occurring. In other words, an increase in the amount of amboceptor in the case of a complex bacteriolysin effects an inhibition apparently comparable to the inhibition of hemolysis produced by an increased dosage of venom.

As strikingly, however, as the above-cited experiments may contra-indicate the presence of a simple toxin and suggest rather that hemolysis by cobra venom is in all cases effected by a complex hemotoxin, there still remains for explanation the fact that the venom, lacking complements of its own and in the absence of extracellular complements, still effects hemolysis of many species of erythrocytes. Otherwise stated, there remains to be explained the production of hemolysis by an amboceptor in the apparent absence of activating complements, and before final conclusions may be drawn that the lysis of the susceptible corpuscles is due to venom amboceptors, the presence of complements capable of activating such amboceptors must be established.

Cobra venom itself contains no such complements, and in the hemolysis of serum-free erythrocytes, the single remaining hypothetical source of complements is in the erythrocytes themselves. If the susceptible corpuscles do not possess an intracellular complement, the conception of an amboceptor structure for the venom constituent which dissolves such erythrocytes in the absence of complements, must be relinquished. If, on the other hand, susceptible erythrocytes do contain an intracellular complement, the phenomena thus far observed are explainable on the basis of a complex venom hemotoxin: The differences in the susceptibility of erythrocytes may then be referred to differences in their complement content and the failure of maximum doses of venom to hemolyze where smaller doses are effective, may find a possible interpretation in the deviation of the intracellular complement by an excess of amboceptors.

[1] *Münch. med. Wchnschr.*, 1901, 48, p. 697.

Fortunately the correctness of the assumption as to an intracellular complement is subject to a direct experimental test. Should the susceptible corpuscles contain a complement capable of activating venom, or, in other words, of forming a complete hemolysin, the addition of these complement-containing corpuscles to venom and non-susceptible corpuscles might be expected to activate the venom amboceptors not only for the susceptible but for the non-susceptible corpuscles as well. As I have shown elsewhere[1] this is exactly the result which occurs. Thus when ox corpuscles are added to a cobra-venom solution, they remain undissolved; but if there also be added guinea-pig corpuscles, not only are the susceptible guinea-pig corpuscles dissolved, but also the non-susceptible ox corpuscles. Otherwise stated, the addition of the susceptible corpuscles includes the addition of a non-lytic activating substance which in conjunction with the venom effects hemolysis of the non-susceptible corpuscles. For this intracellular activating substance I have employed the term endocomplement. That the activation is effected by an intracellular substance rather than by a physiological action of the cell *per se* is guaranteed by the fact that destruction of the integrity of the cell, as by laking, results in no diminution of the activating power. The following table indicates the activation produced by the endocomplement of laked guinea-pig corpuscles when complementing cobra hemotoxin for the otherwise non-susceptible ox corpuscles (Table 7).

TABLE 7.

Endocomplement Activation.

Amount of Endocomplement ($\frac{1}{20}$)* c.c.	1 c.c. 5 per cent Suspension Ox Erythrocytes *a* +0.02 c.c. 1 per cent Cobra Venom	1 c.c. 5 per cent Suspension Ox Erythrocytes *b* Without Cobra Venom
1.0	complete	0
0.75	"	0
0.5	"	0
0.25	trace	0
0.1	0	0

* The endocomplement preparation employed in the quantitative experiments was as a rule obtained as follows: A given amount of full blood was freed of serum by centrifugalization and washing with 0.85 per cent salt solution. The sediment of corpuscles, after washing, was taken up in a given amount of distilled water, either to the original volume of the blood as shed or to a dilution of this volume, designated as $\frac{1}{2}$, $\frac{1}{5}$, $\frac{1}{10}$, $\frac{1}{20}$, etc., endocomplement. This solution of laked blood then received sufficient NaCl to establish an 0.85 per cent content of the same.

[1] *Berl. klin. Wchnschr.*, 1902, 39, pp. 886, 918.

The activating action of the endocomplement demonstrated for guinea-pig corpuscles in the above experiment is likewise present in the case of all the species of susceptible corpuscles. Moreover, the degree of the activating power of the corpuscles of the several susceptible species varies directly as the degree of susceptibility of these corpuscles to cobra venom alone. Thus among the corpuscles which show the greatest activating power are the highly susceptible corpuscles of man and of the guinea-pig, whereas the much less susceptible corpuscles of the rabbit and the horse possess a relatively slight activating action. On the other hand, in contrast to the susceptible corpuscles, the non-susceptible corpuscles as such possess no activating power. In a few exceptional instances the laking of non-susceptible cells liberates an endocomplement not operative in the intact cell; but in general, non-susceptible corpuscles even when laked possess no activating power. The susceptibility of erythrocytes to cobra venom may be seen, therefore, to be determined by the presence or absence within the cell of an activating substance available for reaction with the venom hemotoxin. Where such an endocomplement is available, as in the case of the guinea-pig, the corpuscles are susceptible; where no such endocomplement is available, as in the case of the sheep, the corpuscles are non-susceptible.

The recognition of complement activation as an essential factor in the hemolysis of the susceptible corpuscles makes apparent the fact that the fundamental process involved both in hemolysis of the susceptible and of the non-susceptible corpuscles is the same, namely, the elaboration of a complete hemolysin from a venom constituent and an activating substance, be it intra- or extracellular. There exists no contradiction of the assumption that it is one and the same venom constituent which, when activated, effects hemolysis both of the susceptible and the non-susceptible corpuscles. The following table summarizes a series of combinations which illustrate the activating action of the endocomplement of several species of susceptible corpuscles (Table 8).

Close upon the discovery of the endocomplement attempts were made to determine its more characteristic properties, among others its thermal reactions. It was found that by heating the laked susceptible corpuscles for one-half an hour at 62° C. the entire activating

action of the endocomplement is destroyed. At 60° C. the inactivation is almost complete (Table 9).

This finding, in itself correct, led to the deduction that the endocomplement, not unlike the general class of serum complements, is

TABLE 8.

Summary of Endocomplement Activation of Cobra Venom for Non-susceptible Corpuscles.

Endocomplement from	Erythrocytes of: Ox	Goat	Sheep
Rabbit	+	+	+
Man	+	+	+
Dog	+	+	+
Guinea-pig	+	+	+
Goat	—*	—	—
Ox	+	—	—
Sheep	—*	—	—

* In a single instance activation was observed with each of these combinations. The intact corpuscles of goat, ox, and sheep, however, lack all activating action.

thermolabile—a deduction, however, which further experimentation showed to be incorrect, and for the reasons here given. The endocomplement of susceptible corpuscles is not dissolved in the aqueous menstruum of a laked blood preparation, but remains bound to the

TABLE 9.

Inactivation of Endocomplement in Laked Blood.

Guinea-Pig Endocomplement ($\frac{1}{10}$) c.c.	1 c.c. 5 per cent Suspension of Goat Erythrocytes + 0.02 c.c. 1 per cent Cobra Venom: *a*) Normal Unheated	Heated ½ Hour at: *b*) 60° C.	*c*) 62° C.
1.0	complete	trace	0
0.5	medium	trace	0
0.25	slight	0	0
0.1	faint trace	0	0

suspended stromata of the corpuscles. This fact is determined with especial ease in the case of guinea-pig blood where the stromata are readily isolated by centrifugalization subsequent to the addition of salt, and the following table (Table 10) gives in detail the relative endocomplement content of the menstruum and of the stromata in comparison with that of the laked blood from which both were derived.

From this experiment it is seen that when the stromata are removed from a preparation of laked susceptible corpuscles the remaining

menstruum is devoid of activating action and that the isolated stromata, on the other hand, possess quantitatively the activating power of the original preparation. Furthermore, attempts to destroy this activating action of the isolated stromata by heat revealed the fact that in the absence of the hemoglobin-containing menstruum, no inactivation

TABLE 10.
ACTIVATING ACTION OF STROMATA.

AMOUNT FROM a), b), c) C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES + 0.01 C.C. 1 PER CENT COBRA VENOM +		
	a) Laked Guinea-Pig Blood	b) Hemoglobin-containing Menstruum of Same	c) Stromata of Same
1.0	complete	0	complete
0.5	"	0	"
0.25	"	0	"
0.15	"	0	"
0.1	slight	0	trace
0.05	0	0	0

of the endocomplement occurs at 62° C. This result demonstrated that the endocomplement itself, in contrast to the serum complements, is thermostabile. In view of this fact, the apparent destruction of the endocomplement at 62° C. in laked blood cannot be referred to a thermolability of the endocomplement. It is due, rather, to the complicating presence of the hemoglobin-containing menstruum, as shown by the fact that when stromata are returned to this menstruum and the mixture heated at 62° C., the same complete inactivation of the endocomplement occurs as with freshly laked blood. In illustration of these phenomena the following table suffices (Table 11).

TABLE 11.
HEAT RESISTANCE OF ENDOCOMPLEMENT OF ISOLATED STROMATA.

AMOUNT FROM a), b), c) C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES + 0.01 C.C. 1 PER CENT COBRA VENOM +		
	a Suspension of Guinea-Pig Stromata Unheated	b Suspension of Stromata Heated ½ Hour at 62° C.	c Suspension of Stromata + Menstruum, Heated ½ Hour at 62° C.
1.0	complete	complete	0
0.5	"	"	0
0.25	"	"	0
0.15	"	almost complete	0
0.1	trace	trace	0
0.05	0	0	0

Supplementary to the above experiment it was also determined that when pure hemoglobin is added to the suspension of stromata, the same inactivation of the endocomplement occurs at 62° C. as in the case of laked blood, indicating that in the latter instance the hemoglobin of the menstruum is the constituent which favors the inactivation of the endocomplement. As a result of the higher temperature, it appears that the hemoglobin binds the endocomplement, rendering the latter unavailable for reaction with the venom hemotoxin. In the first publication concerning the endocomplement[1] in which I also described the activating action of lecithin for cobra hemotoxin, the inactivation of the endocomplement in laked blood was taken to mean that the endocomplement is thermolabile and therefore not identical with the intracellular lecithin. The recognition, however, of the fact that the endocomplement is not actually thermolabile but thermostabile, suggested a reconsideration of the possibility of the endocomplement's being lecithin. Were this actually the case, it must then be expected that lecithin heated to 62° C. in the presence of hemoglobin would lose its activating power as does the endocomplement. As a matter of fact this is the result which actually obtains, and the following experiment shows this loss of activating power suffered by a mixture of crystalline horse hemoglobin[2] and of egg lecithin when heated at 62° C. (Table 12).

TABLE 12.

LECITHIN-HEMOGLOBIN INACTIVATION.

AMOUNT OF LECITHIN-HEMOGLOBIN SOLUTION* c.c.	1 c.c. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES + 0.01 C.C. 1 PER CENT COBRA VENOM + Hemoglobin-Lecithin Solution a) Unheated	b) Heated ½ Hour at 62° C.
1.0	complete	0
0.75	"	0
0.5	"	0
0.35	slight	0
0.25	trace	0
0.15	0	0

* Five c.c. hemoglobin solution + 5 c.c. 0.0125 per cent lecithin.

Further, a hemoglobin solution previously heated at 62° C. for one-half hour inhibits the activating action of lecithin, if allowed

[1] Kyes, *Berl. klin. Wchnschr.*, 1902, 39, pp. 886, 918.

[2] This preparation of hemoglobin was kindly furnished by Professor Hübner of Tübingen.

to stand one-half hour at 37° C. with the lecithin prior to the addition of the venom and the erythrocytes.

Additional indications as to the lecithin nature of the endocomplement were also seen in the close correspondence of phenomena exhibited in hemolysis by cobra venom when activated by lecithin and by the endocomplement of laked blood. In both these cases, in contrast to the activation by thermolablie serum complements, the hemolysis occurs at 0° C., the hemolysis is relatively rapid, and lastly, there is marked inhibition of hemolysis by cholesterin.[1] The last point is illustrated by the following experiment (Table 13):

TABLE 13.

CHOLESTERIN INHIBITION OF ENDOCOMPLEMENT AND LECITHIN ACTIVATION.

Amount of Cholesterin Solution c.c.	1 c.c. 5 per cent Suspension of Ox Erythrocytes+0.01 c.c. 1 per cent Cobra Venom+Single Activating Dose of		
	a) Guinea-Pig Endocomplement	*b*) Lecithin	*c*) Guinea-Pig Serum
0.025	0	0	complete
0.01	trace	0	"
0.005	slight	0	"
0.0025	complete	complete	"

The above experiment shows that a given amount of cholesterin which produces no inhibition of the activation by guinea-pig's serum completely inhibits the activating action both of the endocomplement and of lecithin.

All told, the similarities in the action of the endocomplement and lecithin were taken to be highly indicative of an indentity between these substances, but more conclusive evidence was sought by direct chemical methods. The extraction of susceptible erythrocytes with alcohol showed the endocomplement to be quantitatively recovered in this solvent, and when so recovered to retain its activating action on boiling. Further determinations of the solubilities of the endocomplement, moreover, showed it to be soluble in ether and chloroform and insoluble in acetone, corresponding in these properties with lecithin. On the basis of these data, therefore, and in view of the highly specific activating action of lecithin the conclusion was reached

[1] The cholesterin solution employed in this and other experiments, unless otherwise indicated, was prepared as follows: 1 c.c. of a hot saturated methyl alcohol solution of cholesterin was added to 9 c.c. of a hot aqueous 0.85 per cent NaCl solution. This gave a homogeneous suspension of cholesterin somewhat less than 1 per cent in concentration.

that the endocomplement is lecithin.[1] More recently I have confirmed this deduction by determining that the intracellular activating substance, when extracted by alcohol and refined by acetone precipitation from chloroform and ether, not only represents the activating power of the laked blood, but, by weight, possesses the activating power of lecithin and exhibits a phosphorus content of approximately 4 per cent.

Thus far it has been argued that the susceptibility of certain erythrocytes to venom hemolysis is due to the presence of an endocomplement and that this endocomplement is lecithin. From this position the assumption might easily be reached that those corpuscles which are not susceptible to venom contain little or no lecithin and hence their non-susceptibility. This is not, however, a fact, and the susceptibility and non-susceptibility of erythrocytes must be referred to other factors than the absolute *amount* of lecithin contained by the cell. The recognition of these factors requires an analysis of the relation of lecithin to other constituents of the cell.

That lecithin is a constant component of erythrocytes has long since been recognized and for many species the amount of lecithin within these cells has been determined by direct chemical analysis. Concerning the exact distribution of lecithin within the erythrocyte, however, and its relation to other constituents of the cell no data have been available. The stroma of the intact red blood corpuscles is, according to Ehrlich,[2] living protoplasm, and in view of this and of the extensive rôle of lecithin in the metabolism of protoplasm in general, the relation of lecithin to the stroma is of marked importance. The lecithin of the red blood corpuscle appears not to be free within the cell and in this it corresponds with lecithin found in various other sites. The lecithin of egg yolk can be extracted but slightly with ether, whereas the total amount may be recovered by alcohol extraction.[3] The lecithin in this case is for the most part coupled with the vitellin of the yolk forming a globulin-like substance soluble

[1] Kyes and Sachs, *Berl. klin. Wchnschr.*, 1903, 40, pp. 21, 57, 82.

[2] *Charité-Annalen*, 1885, 10, p. 136.

[3] Cf. Hoppe-Seyler, *Handbuch der physiologisch- und pathologisch-chemischen Analyse*, VII. Auflage (H. Thierfelder, Berlin, 1903), p. 157.

in salt solution but precipitable on dialyzing.[1] The lecithin is recovered from this combination only by alcohol extraction whereupon the vitellin undergoes changes, losing its solubility in salt solution. In the case of sera and of blood corpuscles, the lecithin is likewise but slightly extracted by ether, but is recovered *in toto* by alcoholic extraction. The same holds also for the isolated stromata of the corpuscles.

On the basis of the intimate relation of the lecithin to the cytoplasm of the stroma occurs the simplest explanation of the differences in the susceptibility of the various species of erythrocytes. In section I it was shown that certain species of corpuscles have a marked activating action for venom, whereas others entirely lack this action. All corpuscles, however, contain approximately the same amount of lecithin as shown both by the activating action of their alcoholic extract and by direct chemical analysis. But in view of the fact that the lecithin in all cases is bound to other substances of the stroma, it appears that the firmness of this binding varies markedly with the species. Thus in goat's erythrocytes the binding is so firm that the affinity of cobra hemotoxin is insufficient to dissociate the complex and these erythrocytes, therefore, neither are susceptible to venom alone nor activate venom for other corpuscles. On the other hand the lecithin of guinea-pig's corpuscles is more loosely bound to the stromata and is therefore available for activation of the venom. Hence these corpuscles do activate venom for other corpuscles and are themselves susceptible to the action of cobra venom alone. Analogous differences in the relation of lecithin to the proteins of blood sera exist and will be discussed in a later chapter. These differences will also be shown to determine the availability of the lecithin of a serum for venom activation.

Abderhalden and Le Count[2] have suggested in contrast to the above explanation that the non-susceptibility of those corpuscles which are not dissolved by cobra venom alone may be due simply to the presence of an intracellular inhibiting substance (cholesterin) rather than to the particular relation of lecithin to the stroma. This suggestion may be dismissed with the state-

[1] Ebenda, p. 369.

[2] *Ztschr. f. exper. Path. u. Ther.*, 1905, 2, p. 199.

ment that the total alcohol-ether extraction of the non-susceptible corpuscles shows that in all cases the amount of cholesterin present is, relative to the amount of lecithin, insufficient to produce an inhibition. Substances resisting such extraction would at all events be considered an integral part of the stroma to which the lecithin is bound. Noguchi,[1] on the other hand, considers that the intra-corpuscular lecithin is in no case available for activation of venom hemotoxin, but that it is the activation of the venom by fats and fatty acids within the susceptible cell which elaborates the complete lysin. This point of view arises from the failure to differentiate between the direct activation of venom by lecithin and an indirect action of the fatty acids, fats, *et cetera,* to be discussed in section VI.

The many factors which may modify the combination of the lecithin with the stroma complex and thus influence the susceptibility of the cell have not been comprehensively determined. In this connection, however, it is to be noted that Sachs[2] has shown that the corpuscles of fetal ox blood are hemolyzed by cobra venom alone, in contrast to the corpuscles of the adult, and Goebel[3] has shown that corpuscles which are non-susceptible to venom in 0.85 per cent NaCl are readily dissolved by the same when suspended in an isotonic sugar solution.

III.

LECITHIN ACTIVATION.

In the foregoing chapter reference has been made to an activating action of lecithin for cobra venom. It now becomes of importance to discuss this action in detail.

Calmette[4] in 1902 showed that certain sera when heated at 62° C. acquire an activating power for cobra venom, not present in the unheated sera. This indication of a thermostabile serum complement appeared of utmost importance and, extending the findings of Calmette, I was able to determine that all sera possess an activating action when heated at from 65°–100° C., and that the activating action in general is more extensive after heating at the higher temperature.

[1] *Jour. Exper. Med.,* 1907, 9, p. 436.

[2] *Centralbl. f. Bact.,* 1903, 34, p. 686.

[3] *Compt. rend. de la Soc. de Biol.,* 1905, 58, p. 420.

[4] *Compt. rend. de l'Acad. Sci.,* 134, p. 1446.

In view of the extreme stability displayed by the activating substance there appeared the possibility of its isolation by the more usual chemical procedures. A given quantity of serum therefore was added to ten volumes of alcohol and the mixture well agitated. The resulting precipitate was removed by centrifugalization and the alcoholic extract evaporated *in vacuo*. The residue thus obtained was taken up in an amount of 0.85 per cent salt solution equal to the original volume of serum and tested with cobra venom for its activating action. This action was found to be marked and comparable to that shown by the heated serum. To further determine the solubilities of the activating product, the salt solution preparation was then shaken with ether, with the result that the activating substance was transferred to the ether. From several sera it was thus determined that the thermostabile activating substance was constantly present and that this substance was soluble in alcohol and in ether.

Ether soluble substances have long been recognized as widely distributed constituents of sera and chief among these cholesterin, lecithin, fats, and fatty acids. After negative results with cholesterin I found lecithin to possess a constant, characteristic activating action for cobra venom.[1]

As a solvent for the lecithin the purest methyl alcohol was employed, a determination showing that up to 10 per cent this alcohol produces no destruction of red blood corpuscles. From a 1 per cent lecithin solution in this alcohol, dilutions were made in 0.85 per cent salt solution and the activating power of lecithin quantitatively determined. It was found that from 0.0025 c.c. to 0.0035 c.c. of the 1 per cent alcoholic solution (0.000025 gm. of lecithin) was sufficient to activate a given dose of the venom for the hemolysis of 1 c.c. of a 5 per cent suspension of ox or goat corpuscles. The quantitative results are displayed in the following table (Table 14).

The lecithin first employed was that from egg yolk, prepared by Merck of Darmstadt. This preparation was neutral in reaction, soluble in ethyl alcohol, and precipitable from ether by acetone. The latter procedure (Altmann-Henriquez) was employed to obtain a purified product and this product corresponded in its activating

[1] P. Kyes, "Ueber die Wirkungsweise des Cobragiftes," *Berl. klin. Wchnschr.*, 1902, 39, p. 886.

power with the original preparation. A second preparation of lecithin from Riedel of Berlin gave quantitatively the same activation, as did also "agfa" lecithin. Additional preparations of lecithin were available through the kindness of Dr. Bergell of Berlin, Professor

TABLE 14.
LECITHIN ACTIVATION OF COBRA VENOM.

AMOUNT OF LECITHIN 1 PER CENT	0.002 C.C. 1 PER CENT COBRA VENOM+ 1 C.C. 5 PER CENT SUSPENSION OF	
	Ox Corpuscles	Goat Corpuscles
0.005	complete	complete
0.0035	"	"
0.0025	"	medium
0.0015	almost complete	trace
0.001	slight	0
0.00075	0	0

W. Koch of Chicago, and Professor Schulze of Zurich. The preparation furnished by Professor Koch was derived from sheep brain while that prepared by Professor Schulze was from the seeds of leguminous plants. These various lecithins, regardless of their origin and the mode of their preparation, agreed quantitatively in activating power with one another and with the preparations first mentioned. Cephalin from the sheep brain, which according to Koch[1] is a dioxystearyl-monomethyl-lecithin insoluble in alcohol, showed quantitatively the same activating power as the egg lecithin. Cerebrin and protagon, on the other hand, produced no activation (Table 15).

TABLE 15.
COBRA VENOM+CEPHALIN, CEREBRIN, AND PROTAGON.

AMOUNT FROM *a*), *b*), AND *c*)	1 C.C. 5 PER CENT SUSPENSION OX ERYTHROCYTES+ 0.2 C.C. 0.1 PER CENT COBRA VENOM		
C.C.	*a* Cephalin (1 per cent)	*b* Cerebrin (5 per cent)	*c* Protagon (5 per cent)
0.1	complete	0	0
0.05	"	0	0
0.025	"	0	0
0.01	"	0	0
0.005	"	0	0
0.0025	"	0	0
0.001	trace	0	0
0.0005	0	0	0
0.00025	0	0	0
Control without venom:			
0.1	0	0	0

[1] *Ztschr. f. physiol. Chemie*, 1902, 36, p. 134.

It was thus made clear that lecithins in general possess a characteristic power of activating cobra venom for non-susceptible corpuscles. The next step was to determine the effect of lecithin upon the hemolysis of susceptible corpuscles. The results show that in all cases lecithin increases the hemolytic action of cobra venom for susceptible corpuscles or in other words, that a sublytic dose of venom for susceptible corpuscles becomes lytic in the presence of an activating dose of lecithin. The following table is an illustration of this action (Table 16).

TABLE 16.

LECITHIN ACTIVATION OF COBRA VENOM FOR SUSCEPTIBLE CORPUSCLES.

AMOUNT OF COBRA VENOM (1 PER CENT) c.c.	1 c.c. OF 5 PER CENT SUSPENSION OF RABBIT ERYTHROCYTES	
	a) Without Added Lecithin	*b*) +0.2 c.c. 0.01 per cent Lecithin
0.1	complete	complete
0.05	faint trace	"
0.025	0	"
0.01	0	"
0.005	0	"
0.0025	0	"
0.001	0	"
0.0005	0	"
0.00025	0	"
0.0001	0	almost complete
0.00005	0	slight
0.000025	0	faint trace
0.00001	0	0
0.000005	0	0

Analogous experiments with other susceptible corpuscles showed that for each species of susceptible corpuscles as with the rabbit, two lytic doses are to be observed, the one, the minimal amount of venom which alone effects complete hemolysis, the other, the minimal amount of venom which produces lysis when activated by lecithin. The latter dose is the only true indicator of the amount of hemotoxin present in a native venom and the susceptibility of the corpuscles indicated by this dose I have designated as the "absolute susceptibility." The amount of difference between these two doses varies with the several species but in all cases the dose necessary for lysis without lecithin is many times that required when lecithin is added. The "absolute susceptibility" of susceptible corpuscles does not differ in extent from that of non-susceptible corpuscles.

The activating action of lecithin for cobra venom thus being determined both in the case of the susceptible and the non-susceptible

corpuscles, the general extent of this activating power was tested by extending the experimentation to other snake venoms. At once it was found that without exception lecithin produces the corresponding activation of all the venoms tested, namely, those of Bothrops lanceolatus, Trimeresurus anamallensis, Crotalus adamanteus, Trimeresurus riukiuanus, Ancistrodon piscivorus, Bungarus fasciatus, Bungarus coeruleus, Naja haje, and Daboia russellii. From among these venoms, the three following suffice to illustrate the general extent of the activating action of lecithin (Table 17).

TABLE 17.

LECITHIN ACTIVATION OF BUNGARUS, DABOIA, AND CROTALUS VENOMS.

Amount of Venom 0.1 per cent a), b), c) c.c.	1 c.c. of 5 per cent Suspension of Ox Erythrocytes+					
	a) Bungarus fasciatus		b) Daboia		c) Crotalus adamanteus	
	I Without Lecithin	II +0.1 c.c. 0.1 per cent Lecithin	I Without Lecithin	II +0.1 c.c. 0.1 per cent Lecithin	I Without Lecithin	II +0.1 c.c. 0.1 per cent Lecithin
1.0	0	complete	0	complete	0	complete
0.5	0	"	0	"	0	"
0.25	0	"	0	"	0	"
0.1	0	"	0	"	0	"
0.05	0	"	0	"	0	"
0.025	0	"	0	"	0	"
0.01	0	"	0	"	0	"
0.005	0	"	0	"	0	"
0.0035	0	"	0	"	0	"
0.0025	0	almost complete	0	marked	0	almost complete
0.001	0	marked	0	medium	0	medium
0.0005	0	medium	0	slight	0	trace
0.00025	0	trace	0	faint trace	0	faint trace
0.0001	0	0	0	0	0	0

The preceding table shows that whereas no one of the three venoms tabulated produced hemolysis of ox corpuscles when added alone, all of these venoms effected a most extensive hemolysis in the presence of lecithin. Analogous experiments with the other venoms enumerated above gave comparable results. With the exception of those of Bothrops lanceolatus and of Trimeresurus anamallensis, both of which showed a lower hemotoxin content, all of the venoms tested displayed a most striking agreement as to the extent of their lytic action. In fact so strict is this agreement that 0.003 mgm. approximates the minimal lytic dose of each of these venoms when activated by lecithin as indicated above.

In view of the number of venoms tested and the constancy of

the results, the generalization was indicated that the hemotoxins of snake venoms are invariably activated by lecithin. It then became of importance to determine whether or not animal toxins, from sources other than the snakes, are activated by lecithin. Calmette's[1] finding that the acute lethal action of scorpion poison is inhibited by antivenin suggested that certain analogies of structure exist between the venoms and scorpion poison and following this suggestion I determined the hemolytic action of scorpion poison, kindly placed at my disposal by Professor Treub. Other than a very slight hemolysis of guinea-pig corpuscles, this toxin showed no lytic action for washed corpuscles. Upon the addition of lecithin, however, the scorpion poison, like the venoms, displayed a marked hemolytic action, dissolving all species of corpuscles indifferently. The following table shows such lecithin activation of scorpion poison for ox corpuscles, and indicates the hemolytic value of this toxin to be about $\frac{1}{20}$ that of cobra venom (Table 18).

TABLE 18.

LECITHIN ACTIVATION OF SCORPION POISON.

AMOUNT OF SCORPION POISON (0.2 PER CENT) c.c.	1 c.c. 5 PER CENT SUSPENSION OX ERYTHROCYTES	
	Without Lecithin	+0.2 c.c. 0.1 per cent Lecithin
1.0	0	complete
0.5	0	"
0.25	0	"
0.1	0	"
0.05	0	"
0.025	0	"
0.01	0	medium
0.005	0	trace
0.0025	0	faint trace
0.001	0	0

The activating action of lecithin is not therefore to be considered as restricted to snake venoms. It is rather a property bearing relation to toxins in general, at least to those of animal origin. This fact is further illustrated by the more recent findings of Morgenroth and Carpi[2] who have shown that the hemotoxin of bee poison is also activated by lecithin and it is also probable that the hemotoxin of the

[1] *Ann. de l'Institut Pasteur*, 1895, 9, p. 225.

[2] *Berl. klin. Wchnschr.*, 1906, 43, p. 1424.

poison of Trachinus draco is activated by lecithin. Briot[1] describes the activation of this fish toxin by heated horse serum in which case the activating substance is in all likelihood the lecithin of that serum. Friedemann[2] has shown also that the secretion obtained from a pancreatic fistula in the dog, although but slightly hemolytic in itself, is markedly hemolytic in combination with lecithin. This appears as a true lecithin activation; the proof is however less conclusive than in the case of the snake venoms, the scorpion toxin, and the bee poison. At all events sufficient evidence is at hand to show that lecithin as an activator plays an extensive rôle with the animal toxins in general.

The cause of the activating action of lecithin was not to be sought in any lytic power of the lecithin itself for, as shown by the following table, lecithins producing the activation tabulated in the above experiments were devoid of lytic action for washed corpuscles (Table 19).

TABLE 19.

NON-LYTIC ACTION OF LECITHINS.

AMOUNT OF LECITHIN *a*), *b*), *c*), *d*) (1 PER CENT) C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES			
	a) Lecithin	*b*) Lecithin	*c*) Lecithin	*d*) Cephalin
0.2	0	0	0	0
0.1	0	0	0	0
0.05	0	0	0	0
0.025	0	0	0	0
0.01	0	0	0	0

This fact that lecithins may be so isolated as to show no hemolytic action is a point which deserves much emphasis in view of the fact that recent workers[3] have employed preparations of lecithin which produce a considerable grade of hemolysis, which hemolysis they wrongly interpret as an essential property of lecithin bearing a relation to its activating power. There are, of course, commercial preparations of lecithin which exhibit a distinct hemolytic action, in fact most such preparations do, presumably either because of admixtures or because of split products of lecithin itself. Possibly also among the varieties of lecithins (lecithans, Koch) there are those which in themselves

[1] *Compt. rend. de la Soc. de Biol.*, 1902, 54, p. 1197.

[2] *Deutsch. med. Wchnschr.*, 1907, 33, p. 585.

[3] Cf. Noguchi, *Jour. Exper. Med.*, 1906, 8, p. 547.

are hemolytic, but notwithstanding this possibility, I find myself inclined to regard marked hemolysis on the part of a lecithin preparation as indicative of an impure product. Be this as it may, the fact illustrated by the above tables remains, namely, that lecithin which shows no lytic action of its own effects the activation of venoms and the degree of this activation is that of lecithins in general regardless of their primary lytic action. The activating action of lecithin therefore, as previously stated, is not to be referred to its lytic action.

On the other hand, in view of the fundamental researches of Ehrlich and Morgenroth concerning the relationship of complement and amboceptor in serum hemolysis, together with the conception of the amboceptor nature of venom hemotoxins, advanced by Flexner and Noguchi, the preferable working hypothesis for an analysis of lecithin activation appeared to be the supposition that the lecithin as a complement reacts chemically with the venom (amboceptor) to form the complete lysin. Not that in all details the hemolysis is strictly comparable to all serum hemolysis, for it must be recognized that the venom hemotoxin is not bound to the corpuscles with the readiness usually seen in the case of serum amboceptors,[1] and further, that lecithin is not the typical serum complement, but that in the main a chemical interaction between lecithin and the incomplete venom hemotoxin possessed of a haptophore group results in the elaboration of a complete hemolysin comparable to the complement-amboceptor complex as conceived by Ehrlich. That such is the nature of the reaction between these two substances seemed the more probable also in view of the known tendency of lecithin to unite with proteins, sugar, *et cetera* (Henriquez and Bing).

Experimental substantiation of the hypothesis of a chemical reaction between the venom and lecithin was not far to seek. Lecithin in physiological salt solution is readily extracted by ether; that is to say, the great bulk of lecithin, but not all. In other words, the distribution of lecithin between the two fluids follows the general law

[1] The binding of venom to red blood corpuscles as first described by Flexner and Noguchi (*Jour. Exper. Med.*, 1902, 6, p. 277) has been severely questioned by Lamb (*Scientific Memoirs by Officers of the Medical and Sanitary Departments of the Government of India*, N. S., 1905, No. 17) who found no evidence of any binding whatsoever. I have not been able to effect a binding comparable to that seen in the case of the amboceptors of serum hemolysins or as described by Flexner and Noguchi. On the other hand it is possible by using a *highly concentrated solution* of venom (cobra) to bind sufficient hemotoxin to corpuscles to effect their solution upon the addition of suitable complements (cf. Kyes, *Berl. klin. Wchnschr.*, 1902, 93, p. 886).

commonly recognized as "loi de partage." The addition, however, of a suitable amount of cobra venom to the lecithin-containing salt solution, was found to interrupt the action of this law, so that ether extraction of the mixture recovered but a slight amount of the lecithin. Each of two equal volumes (10 c.c.) of salt solution, A and B, containing a given amount of lecithin was shaken out with ether. Prior to the shaking out, however, 1 c.c. of a 0.1 per cent cobra venom solution was added to solution B and both A and B were allowed to stand for one-half an hour at 37° C. The ether extract of each was evaporated to dryness and the respective residues taken up in 10 c.c. of salt solution. The extracted aqueous solutions, and the resuspended ether residues from the same, were then tested with cobra venom and non-susceptible ox corpuscles with the results shown in the following table (Table 20).

TABLE 20.

EFFECT OF VENOM ON THE ETHER EXTRACTION OF LECITHIN FROM SALT SOLUTION.

The complete activating dose of the standard lecithin solution with 0.1 c.c. 0.1 per cent cobra venom = 0.005 c.c. (= 0.025 c.c. of the solutions A and B prior to extraction).

AMOUNT FROM EACH A AND B	1 C.C. 5 PER CENT SUSPENSION OX CORPUSCLES + 0.1 C.C. 0.1 PER CENT COBRA VENOM			
	Solution A Lecithin Alone		Solution B Lecithin + Cobra Venom	
c.c.	I Ether Extract	II Extracted Solution	I Ether Extract	II Extracted Solution
1.0	complete	complete	complete	complete
0.5	"	"	medium	"
0.25	"	"	0	"
0.1	"	0	0	"
0.05	"	0	0	"
0.025	trace	0	0	"
0.015	0	0	0	0

From the above procedure it was seen that the addition of cobra venom to a lecithin solution allowed the ether extraction of but one-twentieth part of the lecithin which was extracted from a similar solution in the absence of the venom. From this it appeared that cobra venom has the power of binding lecithin.

A further point taken to be indicative of a chemical reaction between the venom and lecithin was the difference in the reaction of these substances with blood corpuscles when acting singly and when acting in combination with one another. To offer a greater oppor-

tunity for observing the various stages of the process, conditions were instituted tending to inhibit the rapidity of the reaction. These conditions were a low temperature (0° C.) and dilute solutions. It was found that so slight is the power of the native venom hemotoxin to unite with erythrocytes that under the conditions imposed (two hours' contact in dilute venom at 0° C.) no binding of the venom occurs and lecithin was likewise found not to be bound to the corpuscles. On the other hand, corpuscles added to lecithin + venom at 0° were rapidly dissolved (Table 21).

In view of the fact that neither the venom nor the lecithin reacted singly with the cell, the hemolysis occurring in their combined presence was taken to indicate that these substances even at 0° C. react with one another to form a complete hemolysin possessing a greater affinity for the erythrocytes than that shown by the native venom. That the affinity of the cytophile group of the hemotoxin should be increased by an occupation of another side chain of the molecule, is an easily conceivable chemical result—a result which is seen in the analogous phenomenon of the increase in the affinity of the complementophile group of hemolytic serum amboceptors by the union of the cytophilic group with the cell. In the case of the hemolytic sera an exact parallel also is observed. Ehrlich and Sachs[1] have shown instances in which the occupation of the complementophile group of a serum amboceptor increases the affinity of the haptophore group for the receptor of the cell.

The conception of a chemical reaction between the lecithin and the venom hemotoxin (amboceptor) was found to receive further support also from the quantitative relations existing between these substances. If a reaction occurs between the lecithin and venom as between complement and amboceptor, approximately the same quantitative relations might be expected with lecithin and venom as those observed with the serum lysin constituents. In the case of the serum lysins the experiments of von Dungern,[2] Gruber,[3] and Morgenroth and Sachs,[4] have shown that with relatively greater amounts of amboceptor smaller doses of complement are sufficient to effect

[1] *Berl. klin. Wchnschr.*, 1902, 39, p. 492.

[2] *Münch. med. Wchnschr.*, 47, 1900, p. 677.

[3] *Wien: klin. Wchnschr.*, 15, 1902, p. 387.

[4] *Berl. klin. Wchnschr.*, 1902, 39, p. 817.

TABLE 21.

COMBINED ACTION OF LECITHIN AND VENOM AT 0° C.

Complete lytic dose of 0.1 per cent cobra venom with 0.01 c.c. of lecithin solution = 0.005 c.c.
Complete activating dose of lecithin solution with 0.1 c.c. 0.1 per cent cobra venom = 0.005 c.c.

A

Amount of Venom (0.1 per cent) to Which Corpuscles Were Subjected at 0° C. c.c.	1 c.c. 5 per cent Ox Corpuscles + Decreasing Amounts of Cobra Venom for 2 Hours at 0° C. Corpuscles Then Separated from Supernatant Fluid and Washed. Then 0.01 c.c. Lecithin Solution Added to: I Washed Corpuscles	II Supernatant Fluid Containing Fresh Ox Corpuscles
0.1	faint trace	complete
0.50	0	"
0.025	0	"
0.01	0	"
0.005	0	almost complete
0.0025	0	0

B

Amount of Lecithin Solution to Which Corpuscles Were Subjected at 0° C. c.c.	1 c.c. 5 per cent Ox Corpuscles + Decreasing Amounts of Lecithin Solution for 2 Hours at 0° C. Corpuscles then Separated from Supernatant Fluid and Washed. Then 0.1 c.c. 0.01 per cent Cobra Venom Added to: I Washed Corpuscles	II Supernatant Fluid Containing Fresh Ox Corpuscles
0.075	faint trace	complete
0.05	0	"
0.025	0	"
0.01	0	"
0.0075	0	"
0.005	0	0

C

Amount of Cobra Venom (0.1 per cent) c.c.	1 c.c. 5 per cent Ox Blood + 0.025 c.c. Lecithin Solution + Decreasing Amounts of Cobra Venom at 0° C. for 2 Hours: I Hemolysis then Present	II Corpuscles in Tubes Showing No Hemolysis Separated from Supernatant Fluid and Washed: a) Washed Corpuscles + 0.01 c.c. Lecithin Solution	b) Supernatant Fluid Fresh Ox Corpuscles
0.1	complete	......	......
0.05	"	......	......
0.025	"	......	......
0.01	"	......	......
0.005	faint trace	0	complete
0.0025	0	0	medium
0.001	0	0	0
0....	0	0	0

hemolysis and Neisser and Wechsberg[1] have shown that in the case of certain bacteriolytic sera, an extreme excess of amboceptors may inhibit the action of a minimal amount of complement. Relations analogous to these do exist between venom and lecithin. It was found that with an extreme amount of venom, a greater amount of lecithin is required to effect hemolysis than when less venom is employed. In other words, with a fixed amount of lecithin an increase in the amount of venom beyond a certain point inhibits the lysis. This phenomenon is displayed in the experiment tabulated below (Table 22).

TABLE 22.

QUANTITATIVE RELATIONS BETWEEN COBRA VENOM AND LECITHIN.

Amount of Lecithin Solution 0.2 per cent c.c.	1 c.c. 5 per cent Suspension of Ox Erythrocytes + a) 0.4 c.c. 5 per cent Cobra Venom	1 c.c. 5 per cent Suspension of Ox Erythrocytes + b) 0.1 c.c. 0.1 per cent Cobra Venom
0.05	complete	complete
0.035	medium	"
0.025	slight	"
0.015	faint trace	"
0.01	0	"
0.0075	0	medium
0.005	0	trace
0.0035	0	0

In this experiment it is seen that the same amounts of lecithin produced less hemolysis with an extremely large dose of venom than with $\frac{1}{200}$ of that amount. Also that where the larger dose of venom failed to effect hemolysis with a given amount of lecithin, an increase in the amount of lecithin resulted in hemolysis. This phenomenon is analogous to that observed by Neisser and Wechsberg for the bacteriolytic sera which is explained on the basis of a chemical binding of the complement by an excess of amboceptors which do not enter into relation with the cell receptors. Were the action of the venom that of indirectly rendering the corpuscles susceptible to a lytic action of lecithin as such, an increase in the amount of venom would be expected to increase this susceptibility. Such is not the case. Likewise if the lecithin were assumed to act independently on the cell to make it more susceptible to venom, the susceptibility of the cell would not be expected to be less for the larger dose of venom.

[1] *Münch. med. Wchnschr.*, 48, 1901, p. 697.

The results just discussed are those obtained however only when an extreme excess of venom (amboceptor) is employed. With less excessive doses of the venom it was found that the quantitative relations between lecithin and venom are comparable to those given by Morgenroth and Sachs (*loc. cit.*) for complement and amboceptor in the hemolytic sera; namely, that within limits the larger the dose of venom the smaller the amount of lecithin required to effect hemolysis, and vice versa, that the larger the amount of lecithin, the smaller the amount of venom required. The following table (Table 23) indicates these quantitative relations in detail.

TABLE 23.

QUANTITATIVE RELATIONS BETWEEN COBRA VENOM AND LECITHIN.

A. 1 c.c. 5 per cent Ox Corpuscles		B. 1 c.c. 5 per cent Ox Corpuscles	
Amount of Cobra Venom (1 per cent) c.c.	Amount of Lecithin (0.025 per cent) Required for Activation c.c.	Amount of Lecithin (0.025 per cent) c.c.	Amount of Cobra Venom (1 per cent) Required for Complete Hemolysis
0.01	0.035	0.3	0.00001
0.001	0.05	0.06	0.0001
0.00025	0.075	0.06	0.005
0.0001	0.1		
0.00001	0.5		

From these results it appears that the quantitative relations between lecithin and venom correspond closely with those observed for complement and amboceptor in general and are indicative of a chemical reaction between these substances.

Additional and more complete evidence as to a chemical reaction between lecithin and the venom was found in the fact that when lecithin and venom are mixed and allowed to stand some hours prior to the addition of blood corpuscles, hemolysis occurs much more rapidly upon the addition of corpuscles than when venom, lecithin, and corpuscles are mixed at the same time. When lecithin and corpuscles or venom and corpuscles are similarly mixed, no corresponding elimination of the incubation period results. It appears then that the incubation period represents the time required for a reaction between lecithin and venom and that when a period is afforded for this reaction previous to the addition of blood, the complete toxin is formed (complement-amboceptor complex) so that

upon the addition of the corpuscles the hemolytic action is relatively rapid.

From the total data here indicated the deduction was made that the venom and lecithin react chemically to form a complete hemotoxin and this hemotoxin was designated as "cobra lecithid." The ultimate proof, however, of such a process was recognized to be in the actual isolation of the complete hemotoxin and this proof is advanced in the following section dealing with the production and isolation of cobra lecithid.

IV.

ISOLATION OF COBRA LECITHID.

Attempts to analyze the phenomenon of activation as displayed in serum lysins have been rendered the more difficult by the inadaptability of the lytic substances to direct chemical analysis. The most ingenious of biological experiments have been elaborated to substantiate this or that hypothesis as to the exact mechanism of serum activation, but even where best controlled, the results are to be considered tentative, pending a more satisfactory chemical determination of the reagents participating.

The recognition of the activating action of lecithin for venom hemotoxin afforded the first opportunity of analyzing an activation in which a reagent of known chemical structure was involved. Moreover, the relative stability both of the venom hemotoxin and of the lecithin offered an opportunity for chemical procedure far more favorable than that presented by the serum lysins. Convinced by the biological experiments cited in the previous section that the activation of the venom hemotoxin by lecithin was essentially a chemical reaction between these substances, I therefore attempted the isolation of the assumed end product of this reaction, cobra lecithid, by direct chemical methods.

In the absence of a common solvent for venom and lecithin a reaction between these substances was invited by emulsifying an aqueous solution of venom with a chloroform solution of lecithin. In detail the method as first employed was as follows:[1]

Forty c.c. of a one per cent solution of cobra venom in 0.85 per cent salt solution and 20 c.c. of a 20 per cent solution of lecithin in chloroform were brought together

[1] Cf. Kyes, *Berl. klin. Wchnschr.*, 1903, 42, p. 21.

in a container of about 100 c.c. capacity. This mixture of chloroform lecithin solution and aqueous venom solution was vigorously agitated in a shaking apparatus for two hours. Subsequently to this the resulting emulsion was centrifugalized for from $\frac{3}{4}$ to 1 hour in an electrical centrifuge with an arm length of five inches and showing 3,000 revolutions to the minute. From this centrifugalization there resulted a distinct separation of the aqueous solution from the chloroform solution, the line of separation being sharply marked by a compact, clearly defined intermediate emulsion zone less than 1 mm. in thickness. The supernatant aqueous portion was removed from the underlying chloroform by means of a fine pipette. The chloroform portion was likewise regained

TABLE 24.

CONVERSION OF NATIVE HEMOTOXIN INTO COBRA LECITHID.

1 c.c. 5 per cent suspension ox erythrocytes+0.2 c.c. 0.1 per cent lecithin.

Amount in c.c.	A Native Cobra Venom (Control) 0.001 per cent	B Same Venom Shaken Once with Chloroform Lecithin Solution 0.1 per cent	C Same Venom Shaken Twice with Chloroform Lecithin Solution 1.0 per cent	D Cobra Lecithid Precipitated by Ether from Chloroform Lecithin Solution 0.002 per cent*
1.0	complete	complete	0	complete
0.75	"	"	0	"
0.5	"	"	0	"
0.35	"	"	0	"
0.25	"	"	0	"
0.15	"	"	0	"
0.1	almost complete	"	0	"
0.075	marked	"	....	"
0.05	slight	"	....	almost complete
0.035	trace	almost complete	....	marked
0.025	almost 0	medium	....	slight
0.015	0	slight	....	trace
0.01	0	trace	....	almost 0
0.0075	0	almost 0	....	0
0.005	0	0	....	0
0.0035	0	0	....	0
0.0025	0	0	....	0
0.0015	0	0	....	0
No. of lytic doses computed for total 40 c.c. of original solution	266,000 to 267,000	800	0	266,000 to 267,000
Percentage of hemolysin in each preparation	100	0.3	0.0	100

* Reckoned on the basis of the original aqueous venom solution.

in almost complete amount, from 19 to 19½ of the original 20 c.c. being recovered. Neither of the solutions thus separated differed in optical appearance from the corresponding solution employed in forming the emulsion other than that the aqueous solution was slightly clearer than that first added. The chloroform solution was perfectly clear. Immediately following the separation each solution was tested quantitatively for its hemolytic action. The results showed that 99.7 per cent of the hemolytic power of the original aqueous venom solution had been transferred to the chloroform lecithin solution, the complementary 0.3 per cent being retained by the aqueous solution. The chloroform solution thus known to contain the lysis was next treated with ether in an attempt to isolate the hemolytic substance. Five volumes of chemically pure ether (water-free, distilled over sodium) were added to the chloroform solution with the result that a fairly abundant flocculent precipitate appeared and gradually settled to the bottom of the container leaving an appreciable amount of lecithin dissolved in the supernatant ether-chloroform mixture. Employing the centrifuge this precipitate

was recovered in a compact mass and washed in five volumes of ether by shaking and centrifugalization for from 10 to 20 times to effect a removal of admixed lecithin. The hemolytic power of the precipitate was then tested and proved to be quantitatively that of the chloroform solution prior to precipitation with ether, namely 99.7 per cent of the hemolytic power of the original aqueous venom solution. To make the method quantitatively more perfect the aqueous venom solution which retained 0.3 per cent of its original hemolytic power was treated a second time with chloroform lecithin and the lysin representing this 0.3 per cent precipitated with ether as above. Thus the hemolytic power of the native venom solution was transferred to the chloroform lecithin solution and from this latter solution a substance, cobra lecithid, was isolated which represented quantitatively (100 per cent) the hemolytic power of the original venom solution. Table 24, p. 215, illustrates the hemolytic values at various stages in the procedure as outlined.

In order to determine whether the treatment with chloroform lecithin modified the native venom as a whole or simply its hemolytic principle, the aqueous solution from which the hemotoxin had been completely removed was tested for its neurotoxic action. It was found that the neurotoxin content of the treated venom was exactly that of the same solution before treatment, showing that the lecithin treatment involved the removal of the hemotoxin only. The following table (Table 25) shows the correspondence in toxicity of the venom for mice (15 gm.) before and after the shaking with the chloroform lecithin solution.

TABLE 25.

COMPARATIVE DETERMINATIONS OF NEUROTOXIN CONTENT OF UNMODIFIED AND HEMOTOXIN-FREE COBRA VENOM.

AMOUNT OF 0.01 PER CENT SOLUTION VENOM c.c.	MICE 15 GM. WEIGHT	
	A Unmodified Venom	B Hemotoxin-free Venom
0.5	+ after 2 hours	after 1 hour
0.35	+ " $2\frac{1}{4}$ "	+ " $1\frac{1}{4}$ "
0.25	+ " $1\frac{3}{4}$ "	+ " $1\frac{1}{4}$ "
0.05	+ " $2\frac{1}{2}$ "	+ " 8 "
0.12	+ " 30–40 "	+ " 30–40 "
0.10	lived	lived

From this table it is seen that the neurotoxic action of the venom after treatment with chloroform lecithin was quantitatively the same as prior, in each instance the minimal lethal dose being 0.12 c.c. of a 0.01 per cent solution. In other words, the entire neurotoxic principle of the native venom had been retained whereas the entire hemotoxic principle had been removed. This actual separation of the hemotoxin and the neurotoxin into two solutions without the destruc-

tion of either added direct proof to the correctness of Myers'[1] contention that these two toxic principles of cobra venom are distinct.

The method of isolating cobra lecithid above outlined was that first employed and in the earlier stages of the work gave constant results. At a later period in the investigation, however, difficulty was experienced in transforming the total hemotoxin content of the venom into its lecithid. In certain instances even after weeks, the agitation of a given cobra venom solution with lecithin chloroform resulted in the formation of but a relatively small amount of cobra lecithid. Inasmuch as new supplies of material had been necessary in the course of the investigation this contradiction of the earlier results was attributed to differences in the materials used. It was at first assumed that the causal differences were those existing between the various specimens of cobra venom. Test-tube experimentation, however, showed the latter preparations of venom to be comparable in hemolytic power to those at first employed. The cause of the disparity in the results was then sought in differences in the lecithins, it being determined that commercial preparations of this substance vary markedly, and that a chief point in the variation is the acidity of the product. The effect upon lecithid formation of alkali neutralization of the acid by-products of lecithin decomposition was tested. It was found that the acidity of these by-products inhibits the formation of cobra lecithid and that by the neutralization of the by-products a constant and complete yield of the lecithid could be obtained. This effect of alkali neutralization upon the lecithid production is illustrated in the following experiment (Table 26).

Four hundred c.c. of a 0.5 solution of cobra venom in water were shaken four weeks with 600 c.c. of 20 per cent lecithin chloroform solution. The resulting emulsion was centrifugalized and from the chloroform portion a small sample was removed and tested for its hemolytic action. The mixture was again emulsified and sufficient NaOH was added to half neutralize its acidity as determined by titration. The emulsion was shaken continuously for two days, at the end of which period an amount of HCl equivalent to the NaOH previously used was added,[2] the mixture then centrifugalized, and a second test of the chloroform solution made for its hemolytic power.

The small amount of chloroform lecithid solution removed at each of the two periods for testing was so diluted that it represented a 0.005 per cent solution relative to the native venom employed. The hemolytic power was tested by the addition of decreasing amounts of this dilution to 1 c.c. of a 5 per cent suspension of goat's cor-

[1] *Jour. Path. and Bact.*, 1900, 6, p. 415.

[2] For the elimination of the salts of the fatty acids formed.

puscles. As a control the lytic action was compared with the hemolysis produced by a 0.005 per cent solution of native venom with the same blood unit plus 0.1 c.c. of a 0.1 per cent lecithin solution. The tabulated results are as follows:

TABLE 26.

Amount of 0.005 per cent Solution c.c.	Hemolysis of 1 c.c. of a 5 per Cent Suspension of Goat Erythrocytes		
	A Lecithid in Chloroform Solution after Four Weeks' Shaking without Alkali	B Lecithid in Chloroform Solution after Subsequent Two Days' Shaking with Alkali	C Native Venom Complemented with Lecithin
1.0	complete	complete	complete
0.75	"	"	"
0.5	"	"	"
0.35	marked	"	"
0.25	slight	"	"
0.15	trace	"	"
0.1	o	"	"
0.075	o	"	"
0.05	o	medium	medium
0.035	o	trace	trace
0.025	o	faint trace	faint trace
0.015	o	o	"
0.01	o	o	"

This experiment demonstrated clearly that the alkali neutralization of the acid by-products contained in the venom lecithin mixture removed an inhibition to cobra lecithid formation. Whereas after four weeks' constant shaking but a small portion (approximately 15 per cent) of the hemolytic principle of the venom was found in the chloroform solution, a much shorter time (two days) subsequent to the addition of the alkali sufficed to complete the total transfer of the hemolytic power from the aqueous to the chloroform solution. Therefore taking into account the inhibiting action of acid thus demonstrated the explanation of the inconstant yield of cobra lecithid at times experienced appeared to be in the acidity of certain preparations of lecithin used. At all events the recognition of the inhibiting action of an acid reaction pointed to a modification of the earlier method of lecithid production, which modification resulted in a process giving without fail a 100 per cent yield of cobra lecithid. This process was elaborated as follows:

In order to reduce the time element to a minimum one half neutralization of the acidity of the venom lecithin mixture was effected immediately following rapid emulsification. At once it became apparent, however, that in all such emulsions an increase in acidity accompanied the progress of lecithid production. This constant

increase in acidity, tending to delay lecithid formation, indicated a systematic addition of alkali at subsequent intervals. Therefore immediately after the first neutralization (½) the emulsion was shaken for two hours, its acidity then determined, and this acidity one half neutralized; it was again shaken for two hours, and then one half neutralized, and the procedure of shaking, titration, and one half neutralization repeated until all signs of increasing acidity ceased.

By employing this system of alkali addition it was possible to effect even in the most unfavorable instances a complete yield of cobra lecithid within a single day. In illustration of the details of the method the following experiment is given (including Table 27).

Four hundred c.c. of a half per cent aqueous solution of cobra venom were shaken vigorously with 400 c.c. of a 20 per cent lecithin chloroform solution. From the resulting emulsion 5 c.c. were then withdrawn and the acidity of this sample titrated with $\frac{1}{10}$ normal NaOH.[1] For this neutralization 3½ c.c. of $\frac{1}{10}$ normal NaOH was

TABLE 27.

Amount of Each Solution	Hemolysis of 1 c.c. 5 per Cent Suspension of Goat Erythrocytes by:				
	A Aqueous Portion (0.05 per cent)*		B Chloroform Portion (0.005 per cent)*		C
c.c.	1 Without Lecithin	2 +0.1 c.c. 0.1 per cent Lecithin	1 Without Lecithin	2 +0.1 c.c. 0.1 per cent Lecithin	Native Cobra Venom (0.005 per cent) + 0.1 c.c. 0.1 per cent Lecithin
1.0		faint trace	complete	complete	complete
0.75		" "	"	"	"
0.5		" "	"	"	"
0.35			"	"	"
0.25			"	"	"
0.15			"	"	"
0.1	0		"	"	"
0.075		0	"	"	"
0.05			marked	marked	almost complete
0.035			medium	medium	medium
0.025			trace	trace	trace
0.015			faint trace	faint trace	faint trace
0.01			0	0	0

* The concentration of these solutions is reckoned on the basis of the dried venom used in the original aqueous solution.

required. To the bulk of the emulsion was then added 28 c.c. of normal NaOH or one half the amount of NaOH computed for complete neutralization. After continued shaking of the emulsion for two hours, 5 c.c. were again withdrawn and the acidity determined—2.6 c.c. of a $\frac{1}{10}$ normal NaOH, being required in the titration, indicating the addition of 21 c.c. of normal NaOH to the main bulk of the emulsion for its half

[1] Titration was performed by mixing 5 c.c. of the emulsion with 5 c.c. of amyl alcohol plus 10 c.c. of ethyl alcohol plus three drops of a saturated alcohol phenolphthalin solution.

neutralization. Shaking was resumed for two hours, the then determined acidity of 5 c.c. being 1.9 c.c. of a $\frac{1}{10}$ normal NaOH solution, indicating the addition of 15 c.c. of normal NaOH for one half neutralization of the bulk. Shaking was again continued for two hours, the acidity of 5 c.c. determined to be 1.2 c.c. of a $\frac{1}{10}$ normal NaOH and for half neutralization 10 c.c. NaOH normal were added. Again, shaking was continued for two hours, the acidity for 5 c.c. requiring 1.2 c.c. of $\frac{1}{10}$ normal NaOH solution for neutralization and 10 c.c. of normal NaOH were added for half neutralization. After two hours more of continuous shaking the acidity for 5 c.c. required but 0.6 c.c. of $\frac{1}{10}$ normal NaOH solution for neutralization. In other words the acidity had made no gain during the last period of shaking. An amount of HCl (=84 c.c. normal HCl) equivalent to the total amount of NaOH used up to this point was then added and the emulsion again shaken for two hours. On the following morning the emulsion was centrifugalized and the hemolytic action of the aqueous and chloroform portions determined as indicated in Table 27.

The table just given shows that the entire hemolytic power of the native venom solution was transferred to the chloroform solution and that the lecithid there formed existed as a complete lecithid, that is, one whose lytic action was not increased by the addition of free lecithin.

To isolate the lecithid in substance the 380 c.c. of the chloroform solution recovered in the above procedure were freed from all traces of water by six successive treatments with sodium sulfate and the lecithid then precipitated by the addition of 10 volumes of water-free ether. To effect a complete precipitation the mixture was allowed to stand at $-12°$ C. The resulting white precipitate was washed six times with ether and dried three days *in vacuo* over phosphoric anhydride, sulfuric acid, and paraffin. There resulted 12.4 gm. of substance, somewhat waxy in consistency. This preparation which is comparable to the earliest specimens of cobra lecithid described by me was further purified by dissolving it in 124 c.c. of ethyl alcohol (10 per cent) and by reprecipitation with 10 volumes of ether at $-12°$ C. The resulting precipitate was centrifugalized, washed with ether, and dried to constant weight (10.6 gm.). The lecithid prepared in this manner is snow white and is entirely without the waxy consistency of crude products containing traces of admixed lecithin. This lecithid represents quantitatively the hemolytic action of the chloroform solution from which it was precipitated and therefore that of the original venom solution. Isolated thus in substance the hemolytic action of the lethicid is not in the slightest degree increased by the addition of an excess of free lecithin. This

fact shows the completeness of the reaction afforded by the addition of alkali to the emulsion and contrasts this complete lecithid with other products to be discussed later. The following table in which the concentration of the lecithid solution represents a 0.001 per cent native venom solution indicates the absolute lytic action of the lecithid with and without added lecithin (Table 28).

TABLE 28.

HEMOLYTIC VALUE OF ISOLATED COBRA LECITHID WITH AND WITHOUT ADDED LECITHIN.

Cobra Lecithid c.c.	Hemolysis of 1 c.c. 5 per cent Suspension of Goat Erythrocytes by: a) Cobra Lecithid Alone	b) Cobra Lecithid + 0.1 c.c. 0.1 per cent Lecithin
1.0	complete	complete
0.75	"	"
0.5	"	"
0.35	"	"
0.25	marked	marked
0.15	slight	slight
0.1	trace	trace
0.075	faint trace	0
0.05	0	0

The above table shows that the hemolytic action of the lecithid as isolated is not augmented by added lecithin.

The preparations of cobra lecithid obtained by the last described method in which alkali neutralization is employed are far superior to the earlier lecithid preparations described by me, both from the standpoint of constancy of the yield and the purity of the end product. The chief cause of the difference in the purity of the product is to be found in the fact that in the earlier method of preparation the precipitation of the lecithid from the chloroform solution by ether, carried down a certain amount of admixed lecithin-like substances, the complete removal of which was scarcely possible. Since lecithin itself is ether-soluble these admixed substances which are insoluble in ether may well be considered as hydrates of lecithin. At all events in the last method these admixtures are extensively avoided by the careful dehydration of the chloroform solution with sodium sulfate prior to the ether precipitation of the lecithid. The purification of the precipitate by redissolving in alcohol and reprecipitating with ether is also highly efficient, the admixtures in question appearing never to be precipitated by ether from an alcoholic solution and in this connec-

tion it is also to be noted that the acetone precipitation of lecithin itself varies greatly according to whether the solvent for the lecithin be chloroform or alcohol.

The lecithid as precipitated by ether was found to remain unmodified when allowed to stand under ether, and also to retain its full hemolytic efficiency on drying. The fresh precipitate even when slightly damp with ether was found to be very readily soluble in water giving a perfectly clear light golden solution. Such solutions, however dilute, show a marked tendency to foam when gently agitated. They do not however, even when concentrated, give the biuret reaction. In aqueous solution the cobra lecithid is non-coagulable by heat and retains its hemolytic action on boiling. This thermostability sharply contrasts the hemolysin in this form with the hemolysin in the native venom: Whereas cobra venom in an aqueous solution loses entirely its hemotoxic action when heated for 30 minutes at 100° C., a cobra lecithid solution may be heated six hours at the same temperature without appreciable loss in lytic action. The following table (Table 29) gives the solubilities of cobra lecithid in comparison with those of native cobra venom and of lecithin.

TABLE 29.

Solvent	Cobra Lecithid	Lecithin	Cobra Venom
Water	soluble (30° C.)	insoluble	soluble
Benzol	soluble	soluble	insoluble
Toluol	"	"	"
Chloroform	"	"	"
Alcohol	"	"	"
Acetone	insoluble	insoluble	"
Ether	"	soluble	"
Petroleum ether	"	"	"

From Table 29 it is to be seen that cobra lecithid possesses distinctive solubilities which differentiate it completely as a chemical entity from the two substances employed in its production. The lecithid in contrast to the native venom is soluble in chloroform, benzol, alcohol, and toluol, whereas in contrast to lecithin the lecithid is insoluble in ether and soluble in water. Dissolved in warm water a portion of the cobra lecithid appears on cooling as a microcrystalline white deposit, the individual crystals being transparent and highly refractive. Upon removal of this precipitate the resulting clear solution again becomes clouded by the further formation of crystals.

In a given instance repeated removals of the crystalline deposit reduced the hemolytic power of a lecithid solution to one-third its original value. The sedimented crystals, on the other hand, when washed in cold water and redissolved in warm water displayed quantitatively the hemolytic power lost from the original lecithid solution.

Cobra lecithid possesses no general toxicity. The absence of such an action would be expected from the fact previously stated that the neurotoxin of the aqueous venom solution is not transferred to the lecithin chloroform solution when shaken with the latter. Large quantities of the pure lecithid injected into animals show no lethal action. This fact which I have previously reported[1] has been more recently challenged by Morgenroth and Carpi.[2] These authors find that preparations of cobra lecithid which they isolated possess a lethal action and this action they refer to the presence of a neurotoxin lecithid formed coincidently with the hemotoxin lecithid (cobra lecithid) and supposedly admixed therewith.

It appears to me that the difference in the results obtained by Morgenroth and Carpi and by myself is to be referred to a difference in the preparation of the lecithids tested, and in the experiments of Morgenroth and Carpi I see no proof of the actual formation of a lecithid of the neurotoxin of cobra venom.

A portion of the lecithid employed by Morgenroth and Carpi was prepared by a method essentially different from that used by me in isolating the non-toxic lecithids, and one which is inapplicable where a refined product is desired. The method referred to is the so-called "alcohol method"[3] which I devised early in the lecithid work as a makeshift to be used in those instances where the amount of material was very small and where no attempt was to be made to completely isolate the lecithid but to determine only its presence. Morgenroth and Carpi misapplied this method in attempting the isolation of a pure lecithid by its use. Reference to the method shows that a mixture of venom and lecithin in 50 per cent methyl alcohol is precipi-

[1] *Berl. klin. Wchnschr.*, 1903, 42, p. 956.

[2] *Biochem. Ztschr.*, 1907, 4, p. 248.

[3] In detail the method is as follows: 1 c.c. of a four per cent aqueous solution of venom is added to 1 c.c. of a 20 per cent solution of lecithin and the mixture allowed to stand several hours at 37° C., being subjected to occasional shaking. To the mixture is then added 10 c.c. of ethyl alcohol and the resulting albuminous precipitate is removed. The resulting filtrate is then precipitated with an excess of ether, the precipitate containing the venom lecithid. Cf. Kyes, *Berl. klin. Wchnschr.*, 1903, 42, p. 963.

tated by the addition of ethyl alcohol and that the filtrate thus obtained is then precipitated with ether for a yield of lecithid. In such a procedure it is far from impossible that a small amount of native neurotoxin may remain in the water-containing alcohol sufficiently long to be collected with the ether precipitated lecithid. It is significant that Morgenroth and Carpi obtained their most toxic products by this method.

Likewise the preparations of cobra lecithid which Morgenroth and Carpi isolated by the "chloroform method" are not above the suspicion of containing a sufficient admixture of native neurotoxin to account entirely for their lethal action. When in the "chloroform method" the aqueous solution of native venom is shaken with the chloroform lecithin solution, the emulsion formed may be so fine that traces of the aqueous solution may remain in the latter despite the most persistent centrifugalization, and the substances thus retained appear falsely as chloroform soluble. The precipitation of such chloroform lecithin with ether will then yield a crude lecithid admixed with a certain amount of native venom constituents including neurotoxin. To guard against this occurrence in preparing pure lecithid the chloroform solution after separation by centrifugalization must be thoroughly treated with a dehydrating agent and again centrifugalized.[1] Inasmuch as Morgenroth and Carpi appear to have omitted this step in their procedure, the lecithids which they obtained even with the chloroform method must be considered as somewhat unrefined products, the toxicity of which may well be referred to a content of native neurotoxin.

The fact cited by Morgenroth and Carpi that the admixed neurotoxin is somewhat more thermostabile than native neurotoxin as usually tested is no proof whatsoever that the neurotoxin is present as a lecithid. Sachs and I have emphasized the fact that the heat resistance of venom constituents is greatly modified by the nature of the solvent in which the venom is tested. That the same neurotoxin in a solution of cobra lecithid should show a heat resistance greater than that which it exhibited when tested in water would not be surprising. The same comment applies also to the modification of toxic symptoms which Morgenroth and Carpi cite as an

[1] Cf. use of sodium sulfate in method as given on p. 220.

indication of the transformation of the neurotoxin into a lecithid. These differences are quite as easily explained by the differences in absorption and distribution of the native neurotoxin when injected in a simple aqueous solution and when injected in a solution of cobra lecithid. Then too the reaction of the admixed neurotoxin in Morgenroth and Carpi's preparations with Calmette's serum argues rather against than for its existence in a modified form. According to these authors the admixed neurotoxin reacts with cobra antitoxin exactly the same as does the native neurotoxin of cobra venom.

Finally, however, it is certain that the hemolytic cobra lecithid when properly isolated by the chloroform method contains no traces of a neurotoxic lecithid. Relatively large quantities of the cobra lecithid in aqueous solution produce no general toxic symptoms when injected into animals. Thus an amount of lecithid sufficient to dissolve 200 c.c. of mouse blood was injected into a mouse of 15 gm. weight and produced no symptoms other than an infiltration at the site of inoculation. Likewise 10 c.c. of a one per cent solution of the lecithid injected subcutaneously into a rabbit of 1,750 gm. weight produced no general symptoms but only a circumscribed infiltration of the ventral abdominal wall. More recently also I have injected subcutaneously 1 c.c. of a 10 per cent lecithid solution into each of a series of seven mice without a single lethal result. In view of these facts I can only conclude as previously, that cobra lecithid when actually isolated is non-toxic, and that the results observed by Morgenroth and Carpi may well be referred to admixture of native neurotoxin with the lecithid which they employed.

In addition to its distinctive solubilities and its thermostability cobra lecithid displays in its biological reactions differences which also contrast it with native venom. It is not surprising that the cobra lecithid, formed as it is in the presence of an excess of lecithin, should possess a hemolytic power entirely independent of the lecithin content of the cell upon which it is acting. It will be recalled that the hemolytic action of native cobra venom is in itself limited to those species of erythrocytes which afford intracellular lecithin for the activation of the venom hemotoxin, and that the species of erythrocytes on this basis fall into two classes, the susceptible and the non-susceptible. Not so with the cobra lecithid. The lecithid dissolves all erythrocytes

regardless of species, and to approximately the same extent. The average dose of lecithid necessary for the hemolysis of 1 c.c. of a 5 per cent suspension of erythrocytes is for all species the proportional amount of lecithid formed from 0.003 mgm. of dried venom. It is to be noted that this amount of venom corresponds to the minimal lytic dose required for hemolysis in the test-tube experiments where an excess of extracellular lecithin is used.

A second feature of the lecithid hemolysis which differs from the hemolysis by the native venom is the rapidity of the reaction. When cobra venom is allowed to act on susceptible blood corpuscles a distinct incubation period always elapses before hemolysis occurs. With minimal doses of the native venom the time required for complete hemolysis is from 12 to 18 hours (two hours at 37°, remainder at 8° C.). By the employment of relatively large doses with highly susceptible corpuscles (guinea-pig) the incubation period may be shortened to from 10 to 30 minutes, but even in extreme cases this interval never entirely disappears. Likewise when the minimal lytic dose of venom is activated for non-susceptible corpuscles (goat) by the minimal amount of extracellular lecithin, there must elapse from 16 to 20 hours before the hemolysis is complete. Larger doses of venom and lecithin tend to decrease the incubation period but even under these conditions a distinct incubation period is always to be observed with the native venom. In contrast to this incubation period constantly exhibited by native venom the rapidity of the hemolysis effected by cobra lecithid is marked. In the case of concentrated solutions of the latter the hemolysis is instantaneous. Even where dilute solutions are employed the delay is slight, the maximum being from 15 to 20 minutes. With corresponding doses of the native venom and of the lecithid, where the incubation period with the native venom must be expressed in hours, that with the lecithid is a matter of as many minutes.

The difference in the time required for hemolysis by the venom and its lecithid is of especial importance inasmuch as it shows that the incubation period observed with the native venom is not the time required for the dissolution of the cell by the lysin but rather that it is the time required for the elaboration of the complete lysin from the native hemotoxin and its activating substance—the complete lysin

(lecithid) once formed, there is no delay due to the gradual action of the toxophore group of the toxic complex but the hemolysis is immediate. With this appreciation of the exact nature of the incubation period, the difference between the length of the period, where large and where small doses of venom and lecithin are used, finds its logical chemical explanation in a difference in the rate of lecithid formation in concentrated and in dilute solutions of the substances involved in its production.

An additional important point in which the hemolysis by venom lecithid differs from that produced by the native venom is that whereas the specific antibody (Calmette's antiserum) for cobra venom markedly inhibits hemolysis by the native venom, it possesses no corresponding inhibiting action for the lecithid of the same venom. On the other hand the inhibiting action of cholesterin on native venom hemolysis occurs also with the lecithid hemolysis as shown in the following experiment (Table 30):

TABLE 30.

Cholesterin Inhibition of Cobra Lecithid Hemolysis.

Amount of Cholesterin Solution* c.c.	1 c.c. 5 per cent Suspension of Ox Erythrocytes — 1½ Lytic Doses of Cobra Lecithid
0.1	0
0.075	0
0.05	0
0.035	0
0.025	0
0.015	almost 0
0.01	trace
0.0075	slight
0.005	medium
0.0035	marked
0.0025	almost complete
0.0015	complete
0.001	"

* The cholesterin solution was made by adding 1 c.c. of a hot saturated methyl alcohol solution of cholesterin to 9 c.c. of 0.85 per cent NaCl solution.

The introduction of the technic of ether precipitation from alcohol into the process of cobra lecithid isolation so far enhanced the value of the method for obtaining a pure end product as to allow trustworthy chemical analyses. In these analyses the co-operation of Willstätter and Lüdecke and of von Braun was enjoyed and independent determinations of several preparations of complete lecithid

showed the preparations to consist of one and the same substance. The elementary analysis for nitrogen and phosphorus at the hands of Lüdecke[1] gave the following values:

$$
\begin{array}{ll}
a) & 0.2935 \text{ gm.} = 7.25 \text{ c.c. Nitrogen} \\
 & \underline{0.3018 \text{ gm.} = 0.0625 \text{ gm. } Mg_2P_2O_7} \\
 & N = 2.73 \text{ per cent}; \quad P = 5.76 \text{ per cent} \\
b) & 0.1994 \text{ gm.} = 4.99 \text{ c.c. Nitrogen} \\
 & \underline{0.1859 \text{ gm.} = 0.0403 \text{ gm. } Mg_2P_2O_7.} \\
 & N = 2.8 \text{ per cent}; \quad P = 6.03 \text{ per cent}
\end{array}
$$

The corresponding analysis of another preparation by von Braun gave the following results:

$$
\begin{array}{c}
0.169 \text{ gm.} = 3.89 \text{ c.c. Nitrogen} \\
\underline{0.5118 \text{ gm.} = 0.1045 \text{ gm. } Mg_2P_2O_7} \\
N = 2.84 \text{ per cent}; \quad P = 5.56 \text{ per cent} \\
\text{Hydrogen} = 10.92 \text{ per cent} \\
\text{Carbon} = 59.07 \text{ per cent}
\end{array}
$$

The values for N and P are seen to agree closely in the several determinations and these values correspond with those of a lecithin minus a fatty acid. Thus for a monostearic lecithin Lüdecke gives:

$$N = 2.59 \text{ per cent}; \; P = 5.73 \text{ per cent};$$

and for a monopalmitic lecithin:

$$N = 2.74 \text{ per cent}; \; P = 6.06 \text{ per cent}.$$

The important evidence was thus obtained that in the lecithid formation there is a splitting off of a fatty acid radicle, a fact previously indicated by the increase in acidity of the aqueous chloroform emulsion in the method of preparation above outlined. Further in accord with this finding is also the determination by Lüdecke of the presence of a free fatty acid in the ether employed in precipitating the lecithid from the original chloroform solution.

The direct chemical analyses corroborate, then, the evidence furnished by the distinctive solubilities and biological reactions of cobra lecithid, namely, in showing that this product is a newly formed chemical substance resulting from a reaction between venom and lecithin in which there occurs a splitting of a fatty acid radicle from the latter substance.

[1] Cf. K. Lüdecke, Dissert., Munich, 1905.

The question now logically arises as to whether the lecithid is a synthetic product resulting from the combination of a venom and lecithin quota or whether it represents simply a split product of lecithin resulting from a lipolytic action of the cobra venom: The elementary chemical analyses given above offer no evidence for the determination of this point. The amount of the venom constituent in the lecithid, if such there be, is so slight in comparison with the lecithin quota that its presence is not determined by such chemical methods. On the other hand, as I have elsewhere stated, there are data which strongly favor the assumption that the lecithid is a synthetic product.

Among these data is the fact that the lecithid as a hemolysin possesses an extreme toxicity comparable to that which is constantly noted with the true toxins but which can hardly be ascribed to a monostearyl-lecithin. A proof of greater value, however, and one deserving special emphasis from the biological point of view, is the fact that immunization with cobra lecithid yields an antibody which acts not only upon the lecithid to effect its neutralization but also upon the native cobra venom. The production of such an antibody shows at the same time that the lecithid is a true toxin and also that it contains a quota of the native venom sufficient to stimulate the production of an antibody specific for such native venom.

A series of rabbits were immunized by the subcutaneous injection of increasing doses of cobra lecithid. Prior to the first injection the degree of the normal inhibiting action of the serum (common to all rabbits) was determined for each animal and it was further determined in each instance that this normal inhibiting action was removed by heating the serum for ½ hour at 64°–65° C. The specific neutralizing action of the immune serum was tested quantitatively at various stages in the immunization, the destruction of the non-specific inhibiting action being first effected by heating as above indicated. Not only were the resulting immune sera highly efficient in neutralizing cobra lecithid, but their progression in antibody content was clearly demonstrated. The following table (Table 31) indicates the efficiency of the serum of the same rabbit at four different points during its immunization.

From this table it is seen that a serum which at first had no

specific neutralizing action for the lecithid gradually attained this power as a result of successive inoculations with the lecithid until 0.025 c.c. of the serum sufficed to neutralize completely the lecithid.

TABLE 31.

ANTI-COBRA LECITHID.

AMOUNT OF SERUM ADDED c.c.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES ADDED TO EACH TUBE AFTER SERUM AND LECITHID (2 LYTIC DOSES) HAD STOOD ½ HOUR AT 37° C.				
	I	Serum Drawn at a Period after Inoculation with:			
	Serum Drawn Prior to Inoculation	II 60 c.c. 0.1 per cent Lecithid*	III 85 c.c. 0.1 per cent Lecithid	IV 100 c.c. 0.1 per cent Lecithid	V 200 c.c. 0.1 per cent Lecithid
0.75	complete	0	0	0	0
0.5	"	almost 0	0	0	0
0.35	"	trace	0	0	0
0.25	"	slight	0	0	0
0.2	"	medium	0	0	0
0.15	"	complete	0	0	0
0.1	"	"	0	0	0
0.075	"	"	almost 0	0	0
0.05	"	"	slight	0	0
0.035	"	"	almost complete	almost 0	0
0.025	"	"	complete	slight	0
0.02	"	"	"	medium	almost 0
0.015	"	"	"	marked	trace
0.01	"	"	"	almost complete	slight
0.0075	"	"	"	" "	medium
0.005	"	"	"	complete	"
0.0035	"	"	"	"	marked
0.0025	"	"	"	"	almost complete
0.002	"	"	"	"	complete

* The lecithid used for inoculation was in all instances a complete lecithid which contained neither native venom nor admixed lecithin. The lecithid was boiled prior to inoculation.

The following experiment on the other hand shows the neutralizing action of the same immune serum for native cobra venom (Table 32).

This table shows that the anti-cobra lecithid serum, in addition to neutralizing the lecithid, neutralizes the hemotoxin of the native venom; and not only to an equal but to a greater extent. The reasons for this quantitative difference will be discussed in a later chapter, but at this point it is to be emphasized that the specific antibody obtained by immunization with cobra lecithid reacts with native cobra venom, indicating that the lecithid contains a side chain derived from the venom.[1]

From the standpoint of physical chemistry also the conception of the lecithid as a synthetic product receives substantial support.

[1] For a consideration of the work of von Dungern and Coca in this connection (*Münch. med. Wchnschr.*, 1907, 54, p. 2317; and *Biochem. Ztschr.*, 1908, 12, p. 407) see section VI, p. 272.

Determinations at the hands of Biltz of Clausthal show that the molecular weight of cobra lecithid in chloroform is between 2,000 and 3,000. The molecular weight of lecithin itself is variously determined as between 777 and 807, while Willstätter computes 541 for monostearyl-lecithin. Thus it appears that the lecithid cannot be a split product of lecithin but that a venom constituent with lecithinophile groups must unite many monostearyl-lecithins to produce a body of high molecular weight.

TABLE 32.

Amount of Serum Added	1 c.c. 5 per cent Suspension of Ox Erythrocytes			
	I. Control Normal Serum (Heated ½ Hour 64° C.)		II. Anti-Cobra Lecithid Serum (Heated ½ Hour 64° C.)	
c.c.	With Cobra Lecithid*	With Cobra Venom	With Cobra Lecithid	With Cobra Venom
0.1	complete	complete	0	0
0.075	"	"	0	0
0.05	"	"	0	0
0.035	"	"	0	0
0.025	"	"	0	0
0.02	"	"	almost 0	0
0.015	"	"	trace	0
0.01	"	"	slight	0
0.0075	"	"	medium	0
0.005	"	"	"	0
0.0035	"	"	marked	slight
0.0025	"	"	almost complete	marked
0.002	"	"	complete	almost complete
0.0015	"	"	"	complete
0.001	"	"	"	"

* Twice the lytic dose employed in case of each lysin. Venom complemented with lecithin (0.2 c.c 0.1 per cent).

In view of the results above outlined, I am at present of the opinion that in the formation of cobra lecithid there is a splitting off of a fatty acid radicle and a synthesis of the remaining lecithin molecule with a venom constituent.

Thus far the only lecithid discussed has been that formed with cobra venom. If however the activating action of lecithin in general is to be referred to the formation of lecithids, the isolation of such lecithids should be possible in all instances of a true activation. Such is actually the case. From the full series of toxins mentioned in section as III being activated by lecithin, I have prepared and isolated, in substance, typical lecithids. The general characteristics of these lecithids correspond with those displayed by the lecithid of cobra venom. The quantity of lecithid formed in each instance bears the

same relation to the hemolytic power of the venom, as seen in the case of cobra venom. Thus those venoms which show the same hemolytic power as cobra venom when activated by lecithin in test-tube experiments, produce quantitatively the same yield of lecithid by weight. Further, those venoms (Bothrops and trimeresurus) which showed but a fraction ($\frac{1}{10}$) of the lytic power of the other venoms as tested in the activating experiments, yielded exactly this fraction ($\frac{1}{10}$) of lecithid. Nor is the lecithid formation confined to the snake venoms. Scorpion poison formed quantitatively the quota of lecithid corresponding to its hemolytic power when activated by an excess of lecithin in the test-tube experiments.

The lecithids thus far discussed are those prepared in the presence of an excess of lecithin and are the so-called complete lecithids. In these preparations the lecithinophile groups of the venom hemotoxin are fully saturated. Of equal importance in the analysis of the relation of the venom and the lecithin constituents, however, is the nature of the compounds formed in the presence of relatively small amounts of lecithin—the so-called incomplete lecithids. To obtain products of this sort experimental conditions were so modified that for each gram of cobra venom two grams of lecithin were employed. A one per cent solution of cobra venom was shaken with a 10 per cent solution of lecithin in chloroform as in the perfected method described for the production of complete lecithids, until the production of acid ceased. Provisional tests showed that in this procedure the hemolytic power was not transferred to the chloroform but was retained entirely by the aqueous solution. The complete separation of the resulting viscous emulsion, however, incurred great difficulties. Continued centrifugalization was insufficient and only after many modifications of experimentation was it possible to accomplish this end. Finally however, it was determined that the addition of abundant alcohol and ether served as a simple means for separating the aqueous and chloroform portions of the emulsion. The addition of the ether and alcohol so diluted the chloroform solution and decreased its specific gravity that a spontaneous separation occurred with the aqueous portion subnatant. The details of this procedure are given below:

One thousand five hundred and seventy c.c. of one per cent cobra venom were added to 315 c.c. of 10 per cent chloroform lecithin and shaken until titration showed

no increase in acidity. During the shaking 68 c.c. of n/NaOH were used and at its completion the corresponding 68 c.c. of n/HCl were added. The resulting volume at this stage was 1,993 c.c. (2,000 in round numbers). The required proportion of ether and alcohol necessary to effect separation of the emulsion was then added: ether, one volume (2,000 c.c.); alcohol, $\frac{2}{5}$ volume (800 c.c.). The mixture was then vigorously shaken and allowed to stand over night in a separating funnel. The next morning the aqueous portion was found distinctly separated from the ether-alcohol diluted chloroform portion and was subnatant. The aqueous portion contained a sedimented precipitate. The aqueous portion with its sediment was withdrawn and the sediment removed by centrifugalization. The decanted aqueous portion was then placed at $-12°$ C. with the result that a further precipitate appeared which was removed by centrifugalization. The decanted acqueous portion was again placed at $-12°$ C. for several days, resulting in the further formation of a precipitate from which the supernatant aqueous portion was removed by decantation and then filtered. The several yields of the precipitate were combined, and the aqueous solution (Solution X) showing no further precipitate at $-12°$ C. was preserved for manipulation as hereafter given.

The sedimented precipitate obtained in the above procedure was many times washed with an excess of 30 per cent alcohol at $-12°$ C., preliminary experiments having shown the precipitate to be insoluble under such conditions. The purified product was then regained by

TABLE 33.

Amount of Each Solution c.c.	1 c.c. 5 per cent Suspension Goat Erythrocytes: Substance I (0.01 per cent Solution) a) Without Lecithin	b) +0.1 c.c. 0.1 per cent Lecithin	Cobra Venom (0.01 per cent) + 0.1 c.c. 0.1 per cent Lecithin
1.0	complete	complete	complete
0.5	medium	"	"
0.25	trace	"	"
0.15	0	"	"
0.1	0	"	"
0.05	0	"	"
0.025	0	almost complete	"
0.015	0	marked	"
0.01	0	—	"
0.005	0	—	"
0.0025	0	—	almost complete
0.0015	0	—	marked
0.001	0	—	medium
0	0	0	0

centrifugalization, spread on a porcelain plate, and dried *in vacuo* over sulphuric acid. The product weighed 3.35 gm. This substance, designated as Substance I, reacted slightly acid when suspended in water, and was readily dissolved in warm water upon the addition of a trace of alkali or acid, without reprecipitating on cooling. The clear, slightly alkalin solution gave the biuret reaction and numerous protein precipitation reactions: picric acid, tannic acid, nitric acid,

etc. The addition of an alcoholic cadmium chloride solution caused a voluminous precipitate. Tested for its hemolytic power, Substance I showed the values given in Table 33, p. 233.

This table shows that the hemolytic action of Substance I was increased twenty fold by activation with lecithin but even at that represented but one-tenth the strength of native cobra venom. Consideration of the fact also that but 3.35 gm. of Substance I was obtained as against 15.7 gm. of native venom used in the process showed that the total hemotoxin regained in Substance I was but $\frac{1}{47}$ the total hemotoxin employed. The remaining hemotoxin was therefore sought in the aqueous solution (Solution X) from which Substance I had been originally sedimented and was there found. The hemolytic action of Solution X, as displayed by a small portion withdrawn for the test, is given below (Table 34):

TABLE 34.

QUANTITY IN C.C.	1 C.C. 5 PER CENT SUSPENSION GOAT ERYTHROCYTES		
	Solution X (0.01 per cent)*		Cobra Venom (0.01 per cent) +0.1 c.c. 0.1 per cent Lecithin
	a) Without Lecithin	b) +0.1 c.c. 0.1 per cent Lecithin	
1.0	complete	complete	complete
0.75	"	"	"
0.5	"	"	"
0.35	"	"	"
0.25	almost complete	"	"
0.15	trace	"	"
0.1	0	"	"
0.075	0	"	"
0.05	0	"	"
0.035	0	"	"
0.025	0	"	"
0.015	0	"	"
0.01	0	"	"
0.0075	0	"	"
0.006	0	"	"
0.005	0	almost complete	almost complete
0.0035	0	marked	marked
0.0025	0	medium	medium
0.0015	0	slight	slight
0.001	0	trace	trace
0	0	0	0

* The volume of Solution X as recovered was approximately 2,000 c.c. which in comparison with the original venom solution (1,570 c.c.) represented a $\frac{4}{5}$ dilution. A portion of Solution X further diluted 1:7 (8×) represented therefore a 0.1 per cent venom solution and again diluted ten times as used above, a 0.01 per cent venom solution.

This table shows that the hemolytic action of Solution X was increased 58 fold by lecithin activation and that the total amount of hemolysin contained was approximately that of the original venom

employed. The bulk of Solution X was next treated as follows: By preliminary experiments with small quantities it was determined that by the addition of phenol, an oily, golden-brown diffluent sediment could be obtained from Solution X and that such sediment contained its hemolytic principle in a concentrated form; and further that this separation was best effected when a ½ volume of 5 per cent phenol was added at the temperature of melting ice. On this basis a considerable portion of Solution X was treated in the following manner:

Nine hundred c.c. of SolutionX were shaken vigorously with 450 c.c. of a 5 per cent phenol solution and the mixture removed to a funnel with a sub-attached tube, placed in ice. On the following day the oily sediment was drawn off, the amount recovered being 14 c.c., and rendered clear by the addition of 10 c.c. of alcohol and centrifugalization. The resulting clear fluid was then diluted to 200 c.c. by the addition of alcohol. From this preparation 1 c.c. was immediately taken and diluted with 35 c.c. of physiological salt solution, this giving a dilution which computed relative to the original one per cent venom solution corresponded to a 0.1 per cent venom solution and is designated as "0.1 per cent relative." The hemolytic action of this solution is given in Table 35.

TABLE 35.

QUANTITY IN C.C.	1 C.C. 5 PER CENT SUSPENSION GOAT ERYTHROCYTES +0.1 C.C. 0.1 PER CENT LECITHIN	
	Phenol Sedimented Oil (0.1 per cent rel.)	Cobra Venom (0.1 per cent)
0.0001	complete	complete
0.000075	marked	"
0.00005	medium	"
0.000035	"	"
0.000025	"	marked
0.000015	slight	medium
0.00001	trace	"
0	0	0

Table 35 shows that whereas Solution X contained approximately the full amount of hemotoxin contained by the corresponding venom solution, the oily sediment recovered from Solution X by treatment with phenol contained but ⅓ this amount. In this process therefore a relatively large amount of the hemolysin was destroyed or else evaded sedimentation. But by treating a second portion of Solution X (620 c.c.) as above, an additional 12 c.c. of the oily sediment was obtained and the combined products sufficed for the analyses hereafter referred to. To obtain the hemolysin content of the oily substance, the latter product was placed at $-12°$ C. in a quantity of alcohol amounting to 15 volumes. There resulted a precipitate

which was collected by centrifugalization and washed with alcohol. This product when dried *in vacuo* weighed 0.514 gm. and the hemolytic power of a 0.1 per cent solution of the same is displayed in Table 36.

TABLE 36.

Amount in c.c.	1 c.c. 5 per cent Suspension Goat Erythrocytes		
	Substance II (0.1 per cent)		Cobra Venom 0.1 per cent +0.1 c.c. 0.1 per cent Lecithin
	Without Lecithin	+0.1 c.c. 0.1 per cent Lecithin	
1.0	faint trace	complete	complete
0.5	" "	"	"
0.25	0	"	"
0.1	0	"	"
0.05	0	"	"
0.025	0	"	"
0.01	0	"	"
0.005	0	"	"
0.0025	0	"	"
0.001	0	"	"
0.0005	0	"	"
0.00025	0	"	marked
0.0001	0	"	medium
0.00005	0	"	slight
0.000025	0	medium	"
0.00001	0	"	faint trace
0	0	0	0

This table shows that Substance II although not hemolytic by itself even in large doses was when activated by lecithin tenfold as hemolytic as native cobra venom. This fact itself showed the preparation to be free of lecithin. Nothwithstanding the extreme hemotoxic action of Substance II however, the total hemolytic power of the 0.514 gm. isolated represented but about $\frac{1}{3}$ the hemolytic power of the quantity of Solution X from which the oil was derived, and therefore but $\frac{1}{3}$ of the corresponding amount of the original venom solution.

The filtrate resulting from the precipitation of Substance II from the oil by alcohol was next precipitated with an excess of ether and the sediment isolated—Substance III. This precipitate was voluminous and gelatinous, and soluble in water. In an aqueous solution it was shaken vigorously with ether, regained by sedimentation, and dried *in vacuo* to constant weight. The end product was of a light golden-brown color and weighed 2.3 gm. The hemolytic power is given in Table 37.

From this table it is seen that Substance III possessed some hemo-

lytic action without the addition of lecithin but that with the addition of lecithin, the hemolytic action was 133 times increased, then closely corresponding to that of native venom. In other words the 2.3 gm. of Substance III possessed the same hemolytic power as 2.3 gm. of venom. But inasmuch as the corresponding amount of the original venom solution, of which Substance III was a product, contained

TABLE 37.

AMOUNT IN C.C.	1 C.C. 5 PER CENT SUSPENSION GOAT ERYTHROCYTES		
	Substance III (0.1 per cent)		Cobra Venom 0.1 per cent +0.1 c.c. 0.1 per cent Lecithin
	Without Lecithin	+0.1 c.c. 0.1 per cent Lecithin	
1.0	complete	complete	complete
0.5	"	"	"
0.1	"	"	"
0.05	slight	"	"
0.01	0	"	"
0.005	0	"	"
0.001	0	"	"
0.00075	0	"	"
0.0005	0	medium	marked
0.00035	0	"	"
0.00025	0	slight	medium
0.00015	0	"	"
0.0001	0	"	"
0	0	0	0

11.8 gm. of venom, but 1/5 of its total hemotoxin was isolated as Substance III. In view of the fact that but a very slight hemolytic action was displayed by the aqueous solution derived from Solution X after its treatment with phenol and the removal of the oil, it is evident that in the treatment with phenol a large amount of hemotoxin was either destroyed or so modified as to escape detection. For completeness however about 2,000 c.c. of the aqueous solution above referred to (Solution X) was greatly concentrated by slow distillation *in vacuo* at 50° C. and precipitated with an excess of alcohol. This precipitate, soluble in water, gave the biuret reaction. It was reprecipitated with alcohol and dried *in vacuo*, giving a product (Substance IV) weighing 2.7 gm.[1]

A summary of the above process shows that from a venom solution allowed to react with a relatively small amount of lecithin, the follow-

[1] The filtrate resulting from the alcohol precipitation of concentrated Solution X gave a precipitate on the addition of cadmium chloride. This precipitate, when washed with alcohol and dried, weighed 0.4 gm.

ing substances were differentiated on the basis of their varying solubilities and were isolated:

1. Substance I, appearing as a spontaneous precipitate in the aqueous portion after separation of latter from chloroform portion of original emulsion: Representing $\frac{1}{47}$ of the total hemolytic efficiency of the original venom. Amount actually isolated, 3.35 gm.

2. Substance II, precipitated by alcohol from an oily sediment obtained by treating the aqueous solution with phenol: Representing $\frac{1}{3}$ the total hemolytic efficiency of the original venom. Amount actually isolated, 0.154 gm.

3. Substance III, preciptated by ether from the alcoholic filtrate removed from Substance II: Representing $\frac{1}{6}$ the total hemolytic efficiency of the original venom. Amount actually isolated 2.3 gm.

4. Substance IV, precipitated by alcohol from the aqueous solution resulting after removal of oily sediment from same by phenol treatment: Representing non-hemolytic constituents of the original venom. Amount actually isolated, 2.7 gm.

The chemical analyses of these several products I have given in detail in another place,[1] appreciating, however, that the methods used in their isolation are far less efficient in securing fully isolated substances than in the case of the complete lecithids. The results are sufficient, however, to emphasize the fact that when in the production of a lecithid the amount of lecithin used is insufficient to allow the formation of a complete lecithid hemotoxin, lecithin compounds are formed which differ both from the native venom and from the complete lecithid. Substance III is a body which unlike the native venom is soluble in alcohol, and yet this body represents the venom hemotoxin in a modified form, for when activated by lecithin its hemolytic power is tremendously increased by the formation of a complete lecithid. That there is a lecithin quota in this alcohol soluble hemotoxin compound is shown by its 2.1 per cent phosphorus content.

It is not improbable that a series of incomplete lecithids may be formed differing from one another in the number of lecithinophile radicles of the hemotoxin which are occupied with monostearyl-lecithin. By a method of procedure differing somewhat from that given above but in which also a relatively small amount of lecithin

[1] *Biochem. Ztschr.*, 1907, 4, p. 99.

was employed, I have isolated an incomplete lecithid quite unlike that described above in that it is insoluble in alcohol—a point which serves also to distinguish this latter lecithid from the complete lecithid. On the other hand this lecithid differs from native hemotoxin in that it is non-susceptible to the action of cobra antitoxin (antivenin, Calmette), in this respect coinciding with a characteristic of the complete lecithid. As stated previously, the transformation of the native hemotoxin into the complete lecithid so reduces its affinity for antivenin that this antibody is without inhibiting action upon the complete lecithid. Likewise, the lecithid under discussion, although incomplete, is seen to contain sufficient of the lecithin quota to decrease its affinity for cobra antitoxin to that point where it resists neutralization. The indifference of this incomplete lecithid to Calmette's serum is given in a comparison with native venom in Table 38.

TABLE 38.

Amount of Anti-venom	1 c.c. 5 per cent Suspension of Goat Erythrocytes	
c.c.	Incomplete Lecithid (3 Lytic Doses) +0.1 c.c. 0.1 per cent Lecithin	Cobra Venom (3 Lytic Doses) +0.1 c.c. 0.1 per cent Lecithin
0.05	complete	0
0.025	"	0
0.015	"	0
0.01	"	trace
0.005	"	complete
0	"	"

From Table 38 it is seen that the incomplete lecithid was not neutralized at all by the same cobra antitoxin which completely neutralized the native cobra hemotoxin. It appears then that this lecithid, though far from being a complete lecithid as shown by the character of its hemolysis and its insolubility in alcohol, nevertheless possessed a sufficient quota of monostearyl-lecithin to decrease its affinity for cobra antitoxin in a way comparable to that observed with the complete lecithid.

Substances similar to the incomplete lecithids just described, I have also isolated from the chloroform lecithin solution resulting from attempts to produce a complete lecithid without the addition of alkali. In such instances there are present, in addition to the small

amount of complete lecithid formed, lipoid-hemotoxin compounds which are soluble in chloroform and in alcohol and which are activated by the addition of lecithin in the test-tube experiments.

The existence of incomplete lecithids as a group is strong proof of the synthetic character of the reaction between the venom hemotoxin and lecithin. The existence of an alcohol soluble substance as in the instance first cited, susceptible to activation by lecithin and possessing a relatively high phosphorus content, denotes a modification of the venom hemotoxin in which a phosphorus-containing radicle of lecithin is synthetically involved.

In view of the facts presented in this section concerning the lecithids and more particularly the cobra lecithids, the following conclusions seem justified by the way of summary. The production and isolation of the complete lecithid shows that the activation of venom hemotoxin by lecithin is essentially a reaction between these two substances resulting in an end product which is the actual lytic agent: The splitting off of a fatty acid from the lecithin in the production of the lecithid together with the total absence both of native venom and of lecithin in the end product shows at once that the reaction is a chemical reaction and a non-reversible chemical reaction: The production of a specific antibody to the lecithid by immunization and the reaction of this antibody with native hemotoxin shows at once that the lecithid is a toxin and that it embraces a constituent of the native venom: The chemical analysis on the other hand shows that the lecithid contains also a lecithin constituent: Taken together these facts indicate that the reaction by which the lecithid is formed is a synthesis, which fact is further supported by the occurrence of intermediate products, the incomplete lecithids differing both from the native hemotoxin and from the complete lecithid, but possessing some characteristics of each.

From a general physiological point of view these data concerning the lecithids merit consideration. The transformation of the native venom hemolysins into the fat-soluble lecithids suggests the possibility that many neurotropic toxins, which themselves are insoluble in the tissues of the nervous system, may within the host combine with various lipoids (lecithin, cholesterin, etc.), and thus assuming a lipoid character become the true neurotropic toxins soluble within

the cell. The existence of the incomplete lecithids suggests moreover that for such a transformation the necessary supply of lipoids in the host need not be so great, for in this instance but a relatively small amount of lecithin is required to give the protein constituent certain solubilities of the lipoids.

V.

SERUM ACTIVATION.

The action of normal serum favoring venom hemolysis was first described by Stephens[1] in 1898. This author showed by quantitative experiments that erythrocytes not otherwise dissolved by a given dose of venom in salt solution readily undergo hemolysis in the presence of a non-lytic dose of horse serum. This activating action of horse serum Stephens demonstrated for the venoms of Naja tripudians, Crotalus horridus, Daboia russellii, and Pseudechis porphyriacus.

In 1902 Flexner and Noguchi[2] demonstrated the activating action of a group of sera and interpreted this action as due to serum complements similar to those involved in hemolysis by the complex serum lysins. Flexner and Noguchi were the first to conceive the venom hemotoxins as being of amboceptor structure.

Calmette,[3] later, in 1902, found that certain sera heated at 62° C. showed an activating action for venom and that this property existed irrespective of the power of the serum to activate in the unheated state. From these findings Calmette concluded that the activation of venom could not be due to serum complements as held by Flexner and Noguchi, but that it was dependent rather upon the presence in the serum of a thermostabile "substance sensibilisatrice" (amboceptor). The ability of this substance to unite with the cell Calmette did not test. To explain those instances in which the serum possessed an activating action only after heating, Calmette assumed the presence in the normal serum of a thermolabile antihemolysin which masked the action of the thermostabile "substance sensibilisatrice," and whose destruction by heat removed the inhibition, leaving the "substance sensibilisatrice" free to effect hemolysis in conjunction with the venom.

[1] Thesis, University of Cambridge, November, 1898; also *Jour. Path. and Bact.*, 1900, 6, p. 273.

[2] *Jour. Exper. Med.*, 1902, 6, p. 277.

[3] *Compt. rend. de l'Acad. Sci.*, 1902, 134, p. 1446.

In each of the three investigations cited, the fact was established beyond doubt that certain sera, modified or unmodified, exert a favorable influence on venom hemolysis. Concerning the manner in which such sera exert their influence, no consensus of opinion was reached. The explanation given by Calmette was in two points diametrically opposed to that given by Flexner and Noguchi: Whereas the latter workers considered the venom constituent to be the amboceptor, Calmette attributed this function to a thermostabile substance of the serum; and whereas Flexner and Noguchi contended that normal serum complements activate venom hemotoxin, Calmette denied an activation by such thermostabile substances. The findings as interpreted by these workers appeared mutually excluding.

Extending the work concerning serum activation I have been able to confirm the main findings given by the authors above cited but have found the complexity of serum activation to be far beyond explanation by any of the hypotheses given and indeed beyond explanation by any single hypothesis whatever. There is no general mode of activation which is common to all sera and therefore susceptible to one explanation.

The action of certain sera before and after heating shows clearly that the phenomena observed by Stephens and by Flexner and Noguchi are distinct from those discussed by Calmette. Ox serum, for example, when unheated shows an activation similar to that which Flexner and Noguchi referred to true complements and furthermore this activating action is entirely lost or reduced to a slight trace upon heating the serum as in the activation of true serum complements. But on the other hand if the serum be heated at a higher temperature there appears a second activating action of a different type and comparable to that observed by Calmette. This second activating power may be greater than that possessed by the unheated serum. The two types of activation as displayed by a single serum are shown in the following experiment (Table 39).

This experiment shows that in this instance there are two distinct activating actions, the one which is lost on heating the serum one-half hour at 56° C., and the second which appears on heating the serum at a higher temperature (65° C.). It was found, also, that the substance producing the second activation is far more stable than

indicated by Calmette in that it resists boiling for hours. Indeed the activating action of a serum is often greater when the serum is heated at 75° C. or boiled than when heated at 65° C.

TABLE 39.

Amount of Ox Serum $\frac{1}{10}$ c.c.	1 c.c. 5 per cent Suspension Horse Erythrocytes			
	I Control Ox Serum Alone	II 0.02 c.c. 1 per cent Cobra Venom + Varying Amounts of Ox Serum:		
		a) Normal	Heated ½ hour at	
			b) 56° C.	c) 65° C.
0.5	faint trace	complete	faint trace	complete
0.35	0	almost complete	" "	"
0.25	0	marked	0	"
0.15	0	slight	0	slight
0.1	0	trace	0	trace

The recognition of these two types of activation suggested the investigation of a considerable series of different sera. It was found that according to their activating action when fresh and when heated at various temperatures, the sera of the usual experimental animals fall into five groups. The first of these groups is composed of sera which show an activating action when fresh, when heated at 56° C., and also when heated at from 75° to 100°. Certain other sera which constitute the second group show no activating action either in the fresh state or when heated at 56° C., but only when heated at from 65° to 100°. Still other sera forming a third group show no activation either when fresh or heated at 56° but only on heating at the higher temperatures. The sera constituting the fourth group are those similar to the ox serum in the above experiment in that they activate when fresh, lack an activating power when heated at 56°, but show a second activation when heated at 65° or higher. The fifth group contains the single serum thus far observed which activates in the fresh state only, having no activating action either when heated at 56° or at the higher temperatures.

The combinations in which the above activations were observed are expressed in the following table (Table 40).

From this table it is seen that, with a single exception, all of the sera tested exhibit some grade of activation when heated between

75° and 100°, and furthermore that this activation is independent of the activating power of the same serum in the fresh state.

TABLE 40.

	Activating Power of Serum			Combinations	
	a) Normal	b) Heated at 56°	b) Heated at 65°–100°	Serum	Erythrocytes
I	+	+	+	horse horse horse man rabbit	ox goat* horse man ox
II	o	o	+	man man sheep rabbit	goat* ox sheep* goat*
III	o	+	+	ox sheep	ox ox
IV	+	o	+	guinea-pig ox guinea-pig	ox horse sheep*
V	+	o	o	guinea-pig	rabbit

* Slight hemolysis only, in these instances.

The many differences between the activating action of the several sera as observed in the fresh state, when heated at 56° C., at 65° C., and at 100° C., indicate the multiplicity of factors involved in serum activation considered as a whole. The insufficiency of Calmette's explanation is at once apparent on considering the sera of group I. Horse serum, for instance, shows as a rule the same activating power whether fresh or heated at 56°, whereas according to Calmette's assumption the destruction of the normal anti-hemolysin at 56° should greatly increase the action of the hypothetical thermostabile "substance sensibilisatrice." Also in the case of the sera contained in group II it is apparent that if the assumption were held that there exists a definite thermolabile anti-hemolysin destroyed at 56° C. it must also be assumed that in these particular sera the thermostabile "substance sensibilisatrice" of Calmette is lacking, inasmuch as these sera even when heated at 56° provoke no hemolysis in the presence of venom. Further heating of such sera, however, demonstrates that an activator is actually present. From the total results exhibited in the above table it appears that there is no single definite anti-hemoly-

sin in the sense of Calmette but that in the several sera there are inhibiting substances of varying thermostability whose action is more or less removed by heating the serum at different temperatures. The degree of heating required varies among the different sera and the amount of the activation afforded by the same degree of heat also varies between the several sera.

In view of the stability of the activator encountered in heated sera, the attempt was made to isolate an activating substance from normal serum by direct chemical procedure. To this end a quantity of serum was precipitated with 8–10 volumes of alcohol with the result that the activating substance of the serum was recovered in the alcoholic filtrate. Thus the alcoholic filtrate was evaporated *in vacuo* and the sediment so obtained suspended in a quantity of physiological salt solution equivalent to the original serum employed, with the result that the fluid was found to possess a marked activating action similar to that observed with the filtrate of heat-coagulated serum. Further isolation of the activating substance contained in such a suspension showed it to be lecithin—indeed it was in this connection that the activating action of lecithin was first discovered. The serum precipitate on the other hand when removed from the alcoholic filtrate and redissolved in water showed the distinct power of inhibiting the activating action of the filtrate and this inhibiting action of the precipitate was recognized as due to the serum proteins. Considering, then, the well recognized tendency of lecithin to form protein compounds it appears that in the native serum the lecithin is coupled with proteins, which coupling tends to limit the freedom of lecithin to react with the venom hemotoxin. In fact, in most unmodified sera it appears that no free lecithin exists, at least not in sufficient amount to activate the venom. When, however, factors are introduced, such as heat or alcohol coagulation, which sufficiently modify the proteins, the lecithin is freed from its combination and becomes available as an activator. The varying degrees of heat necessary with the several sera to liberate their lecithin content for activation shows that in the various sera, as in the various species of erythrocytes, the combinations of lecithin with the proteins differ widely. In selected instances, horse serum for example, the lecithin is often so lightly bound that it is at once available for the venom

reaction without the intervention of heat, while in other instances the opposite extreme is met, in that continued boiling barely suffices to liberate the lecithin from its protein compound. Between these extremes many intermediate compounds exist in the various sera, so that the degree of heating required to free the lecithin in a given serum is no criterion as to the degree necessary in a second instance. It seems probable moreover that in the same serum lecithin may be bound not to one protein only but may exist in several combinations of varying stability.

If the above conception of the relation of the proteins to the lecithin content of sera is held to account for the differences in lecithin activation displayed by different sera and by the same serum under different conditions, it might be expected that certain sera whose proteins unite firmly with lecithin would exhibit the power of taking up free lecithin and thus inhibiting its activating action. This is actually the case. Ox serum for example has been observed to completely inhibit the activating action of free lecithin, and in such instances the inhibiting power is fractionally removed by heating the serum at various degrees of temperature which are sufficient to modify and finally coagulate the proteins of the serum.

In view of these facts it appears that the heat modification of sera which effects an increase in their activating action for venom is not the destruction of a definite specific anti-hemolysin labile at 56° or even at 62°, but rather the breaking up at given temperatures of various protein compounds with the resulting liberation of the activating lipoid.

It cannot be maintained that of all lipoids liberated by heat modification of sera, lecithin is the only one which may modify venom hemolysis. It is true, however, that so far as observed, lecithin is the only such substance which effects a *true activation* of a venom hemotoxin. Inasmuch as the lack of recognition of a difference between the activating action of lecithin and that of other lipoids, including some of those of heated sera, is largely responsible for the confusions now existing in the literature concerning venom activation, this difference will be discussed in detail.

Used in its broadest sense the term activation has been employed up to this point to include all instances in which the addition of a

non-lytic dose of a third substance to venom and erythrocytes effects a hemolysis not otherwise occurring. Such a definition includes, therefore, the action of any substance which added to venom and non-susceptible corpuscles produces venom hemolysis. But the many substances which may be employed to this end are separable into two distinct classes according to the general mode of their action, viz.: those which react with the venom to elaborate a complete lysin, and secondly, those which act on the cell to modify its susceptibility.

The activation produced by the first group of substances is a true activation in the narrower sense, and is illustrated by the lecithin reaction with venom resulting in the formation of a lecithid as previously discussed.

In contrast to the true activation there is the activation of the second type which may be designated as indirect or pseudo-activation, in that the so-called activator does not actually enter into the formation of the complete lysin but only indirectly aids in the formation of that substance.

The rôle played by the pseudo-activators in this type of activation is that of rendering the intracellular lecithin of otherwise non-susceptible erythrocytes available for reaction with the venom hemotoxin and thus allowing the formation of the complex lysin—the lecithid.

The susceptibility of erythrocytes to a given venom is not absolute. Whenever the intracellular lecithin of erythrocytes is non-available for reaction with the venom hemotoxin, the erythrocytes are non-susceptible, and vice versa, when the intracellular lecithin is available for such a reaction, the erythrocytes are susceptible. Inasmuch as all erythrocytes contain an activating dose of lecithin, the factor determining their susceptibility is not the amount of lecithin contained, but rather the relation of this lecithin to the cell structure and any factor which may modify this relation may, therefore, modify the susceptibility of the cell to venom. A difference in the susceptibility of the same erythrocytes under differing conditions is illustrated by the following experiment in which the factor determining the susceptibility is nothing more than the menstruum in which the cells are suspended (Table 41).

TABLE 41.

Amount of Cobra Venom 0.1 per cent c.c.	1 c.c. 5 per cent Suspension of Sheep Erythrocytes	
	a) In 0.85 per cent NaCl	*b*) In 10 per cent Cane Sugar
1.0	0	complete
0.75	0	"
0.5	0	"
0.35	0	"
0.25	0	"
0.15	0	"
0.1	0	marked
0.075	0	slight
0.05	0	almost 0
0.035	0	0
0.025	0	0

From the above experiment it is seen that ox erythrocytes, which are never dissolved by cobra venom in physiological salt solution, are highly susceptible to that venom when suspended in a physiological sugar solution. In salt solution the lecithin of these cells is not available for reaction with the venom as it is when the same cells are suspended in sugar solution.

The susceptibility of erythrocytes may also be modified in the *same* menstruum. Those erythrocytes which are non-susceptible in physiological salt solution may be made susceptible in this menstruum by the addition of reagents which so modify the cell that the intracellular lecithin becomes available for a true activation of the venom hemotoxin. As indicated above the substances which act in this way constitute the class of indirect or pseudo-activators.

As early as 1903 in collaboration with Sachs[1] I pointed out the fact that the neutral fats, the fatty acids, and the soaps of the fatty acids all possess in some degree this power of favorably influencing venom hemolysis by modifying the availability of the intracellular lecithin. Repeatedly, however, the pseudo-activation produced by the lipoids has been confused with the true activation by lecithin.

As I previously pointed out, it is a significant fact that the lipoid pseudo-activators themselves are distinctly hemolytic. The hemolytic dose of these substances, moreover, bears a constant relation to the smaller dose required to effect the indirect activation. In other words, the pseudo-activation is produced by the addition of a sublytic dose of a lipoid hemolysin, and it is not surprising that a fraction of the dose which completely destroys the cell should suffice to some-

[1] Kyes and Sachs, *Berl. klin. Wchnschr.*, 1903, 40, pp. 21, 57, 82.

what modify its permeability or the equilibrium of the intracellular constituents.

The hemolytic action of olive oil and the relation of the lytic dose to the dose necessary for indirect activation is shown in Table 42.

TABLE 42.

Hemolysis by Olive Oil, with and without Venom.

Olive Oil (Sat. Methyl Alcohol Solution)* 1/10 c.c.	1 c.c. 5 per cent Suspension Ox Erythrocytes	
	a) Without Cobra Venom	b) +0.2 c.c. 1 per cent Cobra Venom
1.0	complete	complete
0.8	"	"
0.6	marked	"
0.4	0	"
0.2	0	"
0.1	0	"
0.08	0	"
0.06	0	0

* Throughout this and the following experiments with the fatty acids and the soaps the standard solution is a saturated methyl alcohol solution.

From this table it is seen that not only is this fat highly hemolytic but that the dose which is necessary to effect an indirect activation is not such a small fraction of a dose which causes complete destruction of the cell and a much smaller fraction of the dose which sufficiently damages the cell to allow some escape of hemoglobin.

The similar relation between the lytic and the indirect activating doses of a fatty acid is shown in the following experiment (Table 43) in which oleic acid is employed:

TABLE 43.

Hemolysis by Oleic Acid, with and without Venom.

Oleic Acid (Sat. Alc. Sol.) 1/100 c.c.	1 c.c. 5 per cent Suspension Ox Erythrocytes	
	a) Without Venom	b) +0.2 c.c. 1 per cent Cobra Venom
1.0	complete	complete
0.5	"	"
0.25	trace	"
0.1	0	"
0.05	0	"
0.025	0	marked
0.015	0	0

This table shows that the amount of the fatty acid which is required to produce indirect activation with cobra venom is but one-tenth the amount which is required for complete destruction of the cells in

the absence of venom. The factor of the hemolytic dose and the activating dose is 1:10.

The action of the soap of the same fatty acid does not differ markedly from that of the acid itself as shown in the experiment given in Table 44.

TABLE 44.

HEMOLYSIS BY POTASSIUM SOAP OF OLEIC ACID, WITH AND WITHOUT VENOM.

POTASSIUM OLEATE (SAT. ALC. SOL.) 1/1000 C.C.	1 C.C. 5 PER CENT SUSPENSION OF OX ERYTHROCYTES	
	a) Without Venom	b) +0.2 c.c. 0.1 per cent Cobra Venom
1.0	complete	complete
0.5	"	"
0.25	medium	"
0.1	0	"
0.05	0	marked
0.025	0	0
0.015	0	0

This table, when considered in conjunction with Table 43, shows that the factor in the case of the soap approximates that of the fatty acid and that there is here also a distinct relation between the lytic and the activating doses of the lipoid.

For completeness the lytic dose, the activating dose, and the factor displayed by a series of fatty acids and their soaps are given in Table 45.

TABLE 45.

Doses Indicated in c.c. of a Saturated Methyl Alcohol Solution	Palmitic Acid	K Soap of Same	Isotrioxystearic Acid	K Soap of Same	Undecylinic Acid	K Soap of Same	Oleic Acid	K Soap of Same	Eläidic Acid	K Soap of Same	Caprinic Acid	K Soap of Same	Erucic Acid	K Soap of Same
Complete lytic dose without venom	0.1	0.2	0.1	..	0.05	0.07	0.005	0.0005	0.035	0.05	0.035	0.01	0.035	0.05
Complete lytic dose with cobra venom	0.015	0.025	0.015	0.1	0.0075	0.0025	0.0005	0.0001	0.0035	0.001	0.005	0.0025	0.005	0.0025
Factor (approximate)	$\frac{1}{7}$	$\frac{1}{8}$	$\frac{1}{7}$	..	$\frac{1}{7}$	$\frac{1}{4}$	$\frac{1}{10}$	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{50}$	$\frac{1}{7}$	$\frac{1}{4}$	$\frac{1}{7}$	$\frac{1}{20}$

An analysis of the above table shows that a certain variation exists between the factors displayed by the several lipoids, but throughout it is seen that some multiple of the activating dose completely destroys the cell.

Substances other than the lipoids, chloroform, for example, display somewhat the same action, and it is interesting to note that a sublytic dose of tetanus toxin has also been observed to produce an indirect activation of cobra hemotoxin. It would not be strange if ultimately non-lytic substances were found which may sufficiently modify the intracellular equilibrium to effect indirect activation without ever completely destroying the cell. Up to the present, however, I have observed no such substances apart from the various physiological solutions, such as the sugar solution previously considered in this section. With the exception of lecithin such lipoids as possess no hemolytic action show no indirect activating action. On the other hand, it is not of course maintained that a sublytic dose of each and all lysins can produce an indirect activation. The mode of action of the various lysins so far differs that the exact modification of the cell necessary for the liberation of the lecithin from its protein compound occurs only with a certain group of hemolysins. In generalizing then it can be said that a considerable number of lytic substances, when employed in sublytic doses, may so modify a non-susceptible cell as to render it susceptible to venom hemotoxin.

The indirect activation discussed above is entirely distinct from the true activation produced by lecithin. In this latter instance the activating action of the lecithin is in no way dependent upon the lytic action of lecithin as such upon the cell, but rather upon its power to react chemically with venom hemotoxin for the elaboration of the complete lysin—lecithid. In the case of the pseudo-activators, however, no similar end product is formed. Thus when cobra venom is added to a fat, a fatty acid, or the soap of the fatty acid, no chemical reaction occurs which results in the formation of a special lytic substance representing the lytic power of the venom. The mixture of any of these lipoids with cobra venom fails, even on standing, to produce a modification of the rate of hemolysis as compared with that observed when these substances are added coincidently with the blood. There is, in other words, no obliteration of the incubation period such as must result were the action of these lipoids comparable to the true activation produced by lecithin. The degree of heat, moreover, required for the destruction of cobra venom in the presence

of the above lipoids is the same as that required in the absence of these substances, indicating that there is no formation of a thermostabile hemotoxin-lipoid compound, comparable to the lecithid.

In view of these facts concerning the action of the pseudo-activators there seems to be no necessity for further confusion between the indirect action of the lipoids in general with the true activation produced by lecithin.

Now, in the case of heated sera certain lipoids doubtless play a rôle as indirect activators in selected instances; true activation when produced by such sera however is invariably due to their lecithin content. Moreover, the conditions under which a given serum is heated may determine whether the activation which it produces be the indirect activation of the lipoids in general or the true activation by lecithin. Among such conditions are: the degree of heat, the dilution of the serum as heated, the degree of alkalinity of the serum, the freshness of the serum, the amount of free hemoglobin contained, the salt content, etc.

Up to this point the consideration of the activating action of sera has been confined to that of heated sera and it has been shown that the heating which increases the activating action does not produce this change by destroying an anti-hemolysin in the sense of Calmette, but rather by liberating from their protein compounds certain normal lipoid constituents of the serum. Furthermore, it has been shown that the activating action of these free lipoids is of two distinct types, that of all but lecithin being effected by a modification of the cells themselves, whereas that effected by lecithin is a true activation of the venom hemotoxin.

It now becomes of importance to discuss the activating action of fresh unheated sera, and the question at once arises in this connection as to whether or not the activating action of fresh sera may be attributed to the same substances which operate in the case of the heated sera.

Undoubtedly there is a small number of fresh sera whose activation is similar to that produced by the same sera when heated. Such sera are those included in group I, namely, those whose activating action is but slightly, if at all, modified by heating. In a greater number of instances, however, the activation displayed by fresh serum

is susceptible of differentiation from that of heated serum in that the activating substance appears to be thermolabile. An example of such a serum is seen in each of the two following experiments, in which the activating action displayed by the fresh serum is lost on heating at 56° C. for one-half an hour (Tables 46 and 47).

TABLE 46.

Amount of Guinea-Pig Serum $\frac{1}{3}$ c.c.	1 c.c. 5 per cent Suspension of Sheep Erythrocytes		
	a) Normal Serum without Venom	b) 0.02 c.c. 1 per cent Cobra Venom +	
		1) Guinea-Pig Serum, Normal	2) Guinea-Pig Serum Heated ½ Hour at 56° C.
0.5	slight	complete	0
0.25	trace	marked	0
0.1	0	slight	0
0.05	0	trace	0
0.025	0	faint trace	0
0.01	0	0	0

TABLE 47.

Amount of Ox Serum $\frac{1}{10}$ c.c.	1 c.c. 5 per cent Suspension of Horse Erythrocytes		
	a) Normal Serum without Venom	b) 0.02 c.c. 1 per cent Cobra Venom +	
		1) Ox Serum, Normal	2) Ox Serum Heated ½ Hour at 56° C.
0.5	faint trace	complete	faint trace
0.35	0	almost complete	" "
0.25	0	marked	0
0.15	0	slight	0
0.1	0	trace	0

From the above experiments it is seen that, whereas the fresh serum both of guinea-pig and of ox displays a certain activating power for cobra venom, the same serum when heated at 56° loses this activating action. Similar results have been observed also in the following combinations:

Horse	corpuscles	Ox serum
Ox	"	Guinea-pig serum
Sheep	"	" "
Rabbit	"	" "

This possession by fresh sera of an activating power, which disappears on heating at the temperature employed for the inactivation of serum hemolysins in general, would seem to indicate that the

activation produced by these sera is dependent upon true serum complements rather than upon thermostabile activators similar to those discussed above for heated sera.

It must be recognized, however, that the indications afforded by such experiments do not alone constitute absolute proof that even here lecithin may not be the activating substance.[1] There exist in addition, however, certain indications, which taken in conjunction with those of the above experiments seem to show rather conclusively that the activating action of the fresh sera under discussion is not a lecithin activation. These indications are observed in differences which appear in the hemolysis produced by venom when activated by the fresh sera and by lecithin. Thus the rate of hemolysis produced by the serum complements is much less rapid than that occurring with lecithin. In lecithin activation with fairly large doses of cobra venom the hemolysis is immediate, while in the case of the serum activation a considerable incubation period is constant. In the case of lecithin activation, also, the hemolysis proceeds at 0° C. while the activation by the sera under discussion occurs only at a higher temperature.

An additional point which seemingly differentiates the activation by guinea-pig serum for instance from that by lecithin is the difference in the susceptibility of these activators to the inhibiting action of cholesterin. The lecithin activation is markedly inhibited by traces of this substance, whereas the serum activation is but relatively slightly influenced. Table 48 indicates this difference in detail.

This table shows that an amount of cholesterin one hundred times greater than that required to completely inhibit lecithin activation is insufficient to completely inhibit the hemolysis with guinea-pig serum activation.

A somewhat similar differentiation of the guinea-pig serum activation and that of lecithin is furnished also by the following experi-

[1] It might be conceived, for instance, that in such fresh sera, where the lecithin is so lightly bound to the protein that it is free to activate venom, the proper heat modification of the serum might so change the protein lecithin compound as to render the lecithin unavailable for the venom reaction. In fact, in an earlier section of this paper it has been shown that lecithin heated in a solution of hemoglobin is actually so bound to that substance as to lose its activating action, and it was further shown that hemoglobin when heated alone in an aqueous solution acquires the power of inhibiting the activating action of lecithin subsequently added. In view of these facts the simple removal of the activating action of the serum for cobra venom by heat inactivation at 56° C. does not constitute absolute proof that the activating substance itself is thermolabile and hence not lecithin. Indeed a broader statement seems justified, viz., that heat destruction of the activating action of sera in general does not constitute absolute proof that the activating substance itself is thermolabile.

ment, in which an artificial serum, so prepared as to lack an inhibiting action for lecithin activation, still produced a distinct inhibition of the serum activation. The alcohol precipitate of rabbit serum was redissolved in salt solution and the anti-lecithin action of this solution was eliminated by the addition of an excess of lecithin. In other

TABLE 48.
CHOLESTERIN INHIBITION.

CHOLESTERIN SOLUTION* c.c.	1 c.c. 5 per cent Ox Erythrocytes+0.01 c.c. 1 per cent Cobra Venom+Activating Dose of: a) Guinea-Pig Serum	b) Lecithin
0.5	medium	0
0.25	"	0
0.1	"	0
0.05	marked	0
0.025	complete	0
0.01	"	0
0.005	"	0
0.0025	"	complete

* Cholesterin solution made by adding 1 c.c. of a hot saturated methyl alcohol solution of cholesterin to 9 c.c. of 0.85 per cent salt solution.

words, the serum thus prepared, far from possessing an anti-lecithin action, contained sufficient free lecithin to produce a lecithin activation, when employed in sufficient amount. This serum, however, possessed the same power of inhibiting guinea-pig serum activation as did a portion of the similarly prepared serum, to which however no lecithin was added. The details of this experiment with the tabulated results are given below (Table 49).

Twenty c.c. of rabbit serum were precipitated with 180 c.c. of absolute alcohol, the precipitate recovered quickly, and redissolved in 20 c.c. of salt solution. This solution

TABLE 49.

AMOUNT OF INHIBITING SOLUTION c.c.	HEMOLYSIS ALLOWED BY a) Inhibiting Solution without Lecithin*	b) Inhibiting Solution +Lecithin*
0.1	faint trace	complete
0.5	trace	almost complete
0.25	slight	medium
0.15	"	slight
0.1	medium	medium
0.05	"	"
0.025	marked	marked
0.01	"	almost complete
0	complete	complete

* 0.25 c.c. of guinea-pig serum and the inhibiting solution were allowed to stand three-quarters of an hour in contact at 37° C. prior to the addition of 0.01 c.c. 1 per cent cobra venom and 1 c.c. 5 per cent suspension of ox erythrocytes.

showed an inhibition of venom hemolysis both by lecithin activation and by guinea-pig serum activation.

Four c.c. of the inhibiting solution were then allowed to stand three-fourths of an hour with 2 c.c. of a 0.17 per cent lecithin solution. This mixture was then seen to possess a sufficient excess of free lecithin to activate cobra venom when sufficiently large doses of the former were employed. Smaller doses on the other hand sufficed to inhibit the activation by guinea-pig serum to the same degree as the original solution without adding lecithin.

In the above experiment it is seen that an artificial serum, which possessed no anti-lecithin action, still produced distinct inhibition of the fresh serum activation.

A further indication that the activation by the fresh sera under discussion is not due to their lecithin content is the fact that such sera possess the power of actually inhibiting the action of free lecithin. Of the same guinea-pig serum which produces activation in optimum doses, smaller doses suffice to inhibit totally the activating action of a dose of free lecithin. Such a result is seen in the following experiment (Table 50):

TABLE 50.

INHIBITION OF LECITHIN ACTIVATION BY ACTIVE GUINEA-PIG SERUM.

Amount of Guinea-Pig Serum c.c.	1 c.c. 5 per cent Suspension of Ox Erythrocytes + 0.001 c.c. 1 per cent Cobra Venom + 0.075 c.c. 0.025 per cent Lecithin*
0.5	complete
0.25	marked
0.1	trace
0.05	0
0.025	0
0.01	trace
0	complete

* Lecithin and serum allowed to stand one-half hour at 37° C. prior to addition of venom and erythrocytes.

It is scarcely conceivable that the same serum which inhibits lecithin activation should produce a lecithin activation.

The fact that papäin digestion of the sera under discussion destroys their activating action and on the other hand does not modify the activating of lecithin is an additional point which indicates that it is not the lecithin content of these sera which gives them their activating power. Digestion of 5 c.c. of guinea-pig serum with 1 c.c. of 10 per cent papäin solution for one and one-half hours according to the procedure introduced by Ehrlich and Sachs, resulted in the

complete destruction of the activating action of the serum. Table 51 shows the activating action of the same guinea-pig serum before and after such papäin digestion.

TABLE 51.

Amount of Serum	1 c.c. 5 per cent Suspension of Ox Erythrocytes +0.02 c.c. 1 per cent Cobra Venom+Guinea-Pig Serum	
c.c.	a) Normal	b) Digested with Papäin
0.5	complete	almost 0
0.35	"	" 0
0.25	almost complete	" 0
0.15	marked	" 0
0.1	"	0
0.075	medium	0

TABLE 52.

Amount of Lecithin Solution	1 c.c. 5 per cent Suspension of Ox Erythrocytes +0.02 c.c. 1 per cent Cobra Venom+0.025 per cent Lecithin Solution	
c.c.	a) Untreated	b) Digested with Papäin
0.25	complete	complete
0.15	"	"
0.1	"	"
0.075	trace	trace
0.05	0	0

From Table 51 it is seen that the activating action of the guinea-pig serum was destroyed by papäin digestion whereas Table 52 shows that lecithin when subjected to the same treatment retains its full activating power. Similar results are obtained also in parallel experiments in which treatment with HCl and with NaOH is substituted for the papäin digestion.

Although the proof is not to be considered absolute, such findings as those referred to above indicate strikingly the probability that the activation by such sera as are inactivated at 56° C. is not due to their lecithin content.

The question now arises as to the manner in which these sera do effect venom activation and at the outset it should be stated that no consensus of opinion has yet been reached which justifies a definite answer to this question. Assuming for the present, however, that the hemotoxin which is activated by the serum complement is the same as that activated by lecithin, it is at once apparent that the activating action of the fresh serum may be either direct or indirect; in

other words, that the thermolabile activating substances react with the hemotoxin itself or that they act upon the cell to liberate endocomplements. Although this question, as to whether the activating action of fresh sera is a direct or indirect activation, is not definitely answered by such data as are now available, the weight of evidence appears to me to favor the view that the activation is indirect.

Flexner and Noguchi,[1] in their early work concerning serum activation, took the position that a given venom contains "several or many intermediary bodies" and that "these bodies show specific affinities for certain (serum) complements" (p. 288). According to this scheme it was assumed that given corpuscles bound certain venom amboceptors and that these amboceptors were then susceptible to activation by specific serum complements. In other words, a true activation of the venom hemotoxin by serum complements was assumed to occur after the manner of the complement-amboceptor reaction of the true serum hemolysins.

The experiments of Flexner and Noguchi, which at first appeared convincing, do not however, in the light of subsequent work, justify the closeness of the analogy which these authors drew between the activating action of sera in the case of the complex serum lysins and in the case of the venom hemotoxins. It is not meant to argue that venom hemotoxins are no longer to be considered as amboceptors, but rather to show that the early experiments given as proof of a true activation of venom hemotoxin by thermolabile serum complements are inconclusive and in part unreliable. The findings of Kyes, Lamb, and Sachs all show that there is no selective binding of venom amboceptors by the various species of washed erythrocytes which justifies the assumption of "several or many intermediary bodies"—the binding power of the erythrocytes is far too slight to allow a differential determination of multiple hemotoxins in a given venom as attempted by Flexner and Noguchi. As regards the experiments which Flexner and Noguchi advanced to show a true activation of venom amboceptors by serum complements, it is to be remembered that throughout these experiments Flexner and Noguchi proceeded under the false impression that venoms alone lack the power of dissolving erythrocytes and that, as a result, the

[1] *Jour. Exper. Med.*, 1902, 6, p. 277.

bulk of the experiments were performed with erythrocytes which are themselves susceptible to venom alone. The controls given in these instances are insufficient to justify any conclusion as to the rôle of the sera employed.[1] In view of these facts the work of Flexner and Noguchi is insufficient as proof either of a multiplicity of hemolytic amboceptors in a given venom or of a reaction between the thermolabile complements of sera and venom hemotoxin analogous to that observed with the complement-amboceptor complex of the serum hemolysins.

More recently and from a point of view differing somewhat from that of Flexner and Noguchi, von Dungern and Coca[2] also have attempted to demonstrate a multiplicity of cobra venom hemotoxins and a true serum complement activation of this venom. These authors maintained that there are two distinct types of hemotoxin in cobra venom and that one of these is activated by serum complements whereas the other is not. The hemotoxin which von Dungern and Coca conceived as being activated by serum complements they considered as a true amboceptor readily bound to the erythrocytes, whereas the other hemotoxin which is activated only by lecithin they refused to accredit as an amboceptor in any sense of the term.

Von Dungern and Coca treated non-susceptible erythrocytes (ox) with a strong venom (cobra) solution and after removal of the venom solution determined the hemolysis occurring upon the addition of guinea-pig serum and upon the addition of lecithin to the treated corpuscles. They found that the addition of guinea-pig serum resulted in hemolysis, whereas the addition of lecithin produced no hemolysis. Then testing the venom solution with which the corpuscles had been treated, for its power to dissolve normal erythrocytes both in the presence of lecithin and of guinea-pig serum, they found that hemolysis resulted in both instances. It was from these findings that von Dungern and Coca made their deduction as to the presence of multiple hemotoxins in cobra venom and the true serum activation of one of these.

Briefly stated, the results given by von Dungern and Coca are inconstant and when actually occurring indicate conclusions quite

[1] Cf. experiments, *op. cit.*, p. 288.

[2] *Münch. med. Wchnschr.*, 1907, 4, p. 2317.

the opposite of those cited. Neither the tests with the erythrocytes nor those with the supernatant venom solution demonstrate the specific binding of an amboceptor or the true acitvation of such an amboceptor by a serum complement. The simple fact that the supernatant venom from which the amboceptor was supposedly removed by the corpuscle showed hemolysis when tested with fresh corpuscles and guinea-pig serum indicates at once that von Dungern and Coca did not, as they claimed, effect the complete separation of two distinct hemotoxins. The venom solution in question retained after treatment with the corpuscles its power to effect hemolysis not only with lecithin but with the serum as well. It is significant that just in this connection von Dungern and Coca omitted the quantitative details in their experiments and casually referred the hemolysis produced with guinea-pig serum to the activating action of the lecithin content of the serum. Fortunately however Sachs[1] has furnished details in this regard and in repeating von Dungern and Coca's experiments showed that the hemolytic power of the venom with guinea-pig serum is practically the same subsequently to the treatment of the venom with ox corpuscles as prior.

In view of the fact that von Dungern and Coca found the venom which they removed from the ox corpuscles in their experiment still to contain a hemotoxin susceptible to activation by guinea-pig serum and further in view of the fact, as shown by Sachs, that the amount of such hemotoxin is practically the same as that in the original venom solution, the claim by von Dungern and Coca that they actually accomplished a complete separation of two hemotoxins in cobra venom is without force. In fact, influenced by the results of Sachs and by a repetition of their own experiments von Dungern and Coca have themselves more recently relinguished their claims both as to a multiplicity of hemotoxins in cobra venom and as to a true activation by serum complements.[2]

A consideration then of the data thus far accumulated fairly allows the statement that there exist at present no proofs that serum complements other than lecithin react with the venom hemotoxin to effect a true activation.

[1] *Münch. med. Wchnschr.*, 1908, 55, p. 437.

[2] *Biochem. Ztschr.*, 1908, 9, p. 407.

The above statement is far from saying, however, that active sera have no influence on venom hemolysis or that non-susceptible corpuscles are indifferent to treatment with concentrated venom solution. Early in my work with the venoms I found that non-susceptible erythrocytes when treated with concentrated cobra venom were more readily dissolved by complement-containing sera than were unheated corpuscles and it was this finding which led me to accept for a time the opinion held by Flexner and Noguchi that a true activation of the venom amboceptors was effected by serum complements. Later in the work, however, when recognizing more fully the intracellular activating action of lecithin and the extensive rôle played by lytic substances as indirect activators, it appeared to me that the phenomena observed with active sera did not require the interpretation given by Flexner and Noguchi, but that an indirect action of the serum favoring intracellular lecithin activation was more probable. At present I am more convinced that such is the case.

Earlier in the present section I have shown that a considerable number of lytic substances, when employed in sublytic doses and in conjunction with cobra venom, effect a hemolysis not otherwise occurring. This indirect activation has been referred, moreover, to a modification of the erythrocytes rendering the intracellular lecithin available for reaction with the venom hemotoxin.

Now it may be stated that with but a few, if indeed with a single exception, those sera which favorably influence venom hemolysis when fresh and which lose this power when inactivated, possess in some degree a lytic action of their own for the corpuscles tested. Moreover, the degree of the influence of such active sera upon venom hemolysis is in general directly proportionate to the hemolytic power of the serum itself in the absence of venom. Thus the serum most extensively used for the demonstration of an activation is that of the guinea-pig, employed in conjunction with ox corpuscles, and this serum invariably shows a distinct lytic action of its own for those corpuscles (Table 53).

It will be observed in Table 53 that whereas 0.5 c.c. of the serum was required to effect complete hemolysis in conjunction with the venom, the same amount even without venom produced a trace of

hemolysis. The factor between the lytic and the activating doses cannot be given in the above experiment on the basis of the complete lytic dose but it is to be noted that the factor indicated by the maximum non-lytic dose is well within the range of those factors observed with the lipoid indirect activators. Thus whereas 0.1 c.c. was the maximum non-lytic dose with the venom, amounts of serum above 0.25 c.c. sufficed to effect some grade of hemolysis in the absence of venom. Based upon the "nil" dose the factor in this instance is 1/2.5. Comparable results are constantly observed in all cases where the above combination is employed. (Cf. Kyes, Sachs, and von Dungern and Coca.)

TABLE 53.

GUINEA-PIG SERUM ACTIVATION.

Guinea-Pig Serum c.c.	1 c.c. 5 per cent Suspension Ox Erythrocytes	
	a) Without Venom	b) +0.2 c.c. 0.1 per cent Cobra Venom
1.0	slight +	complete
0.75	slight −	"
0.5	trace	"
0.35	faint trace	almost complete
0.25	0	marked
0.15	0	trace
0.1	0	0
0.075	0	0

Likewise in the combination of other sera with other corpuscles a similar lytic action is to be observed. Thus in experiments showing the influence of ox serum on sublytic doses of cobra venom for horse corpuscles a lytic action of the serum for these corpuscles is seen (Table 54).

TABLE 54.

Ox Serum c.c.	1 c.c. 5 per cent Suspension of Horse Erythrocytes	
	a) Without Venom	b) +Sublytic Dose of Cobra Venom
1.0	complete	complete
0.5	"	"
0.25	almost complete	"
0.1	slight	"
0.05	faint trace	"
0.025	0	marked
0.01	0	0

In the above experiment, as in the previous one, it is seen that the amount of serum required to effect complete hemolysis with the venom

not only modified the cell in the absence of the venom, but sufficiently so as to allow the escape of hemoglobin.

Further striking indications of the dependence of the activating action of the serum upon its lytic action are also to be found in the work of von Dungern and Coca. Although the significance of their results was overlooked by these authors, the protocols show that three of the four rabbit sera which they tested and which produced no activation of cobra venom showed also no lytic action of their own in the absence of venom. On the other hand, a fourth rabbit's serum which these authors found to possess an activating power for the venom is seen to possess a hemolytic action of its own. In other words, the sera which showed no lytic action in the absence of the venom showed no activation in the presence of the venom. And, vice versa, the serum which showed a lytic power of its own did produce an apparent activation in the presence of venom.

Now in the absence of actual proof as to a true activation of venom hemotoxin by thermolabile complements and in view of the fact that lytic substances in sublytic doses are known to modify the availability of intracellular lecithin for venom, the above considerations strongly indicate, at least, that the activation of venom by the active sera under discussion is a pseudo-activation, in which the complex serum lysins (complement-amboceptor complex) act simply after the manner of the lipoid lysins in that they indirectly facilitate a reaction between the intracellular lecithin and the venom hemotoxin.

The above explanation accounts, however, only for those instances in which the serum, the venom, and the corpuscles are coincidently present. It is not applicable to those instances where corpuscles treated with venom show an increased susceptibility to active sera. Although the results are not constant, it is nevertheless true that certain non-susceptible corpuscles when treated with concentrated venom in salt solution and when removed from such a solution may be dissolved by an amount of serum which was non-hemolytic for the same corpuscles prior to such treatment. Even here, however, it is not necessary to assume, as was first done, that there is a binding of a venom constituent to the corpuscles and that this constituent is then activated by a serum complement.

It cannot rightly be assumed that those corpuscles which show no

hemolytic reaction with the venom in salt solution therefore suffer no structural modification. It is rather to be remembered in this connection that the venom is a composite of toxic principles, of which the hemotoxin is but one, and further that these same cells when suspended in a physiological sugar solution instead of the salt solution are completely destroyed by the venom. From this point of view it does not appear improbable that the treatment of the corpuscles with the strong venom in salt solution, although not damaging the cell to the extent of allowing hemolysis, does, however, produce profound structural modifications and that these destructive changes so decrease the resistance of the corpuscles as to allow their complete destruction by smaller doses of the complex serum lysins than required in the case of the untreated corpuscles. This explanation of the phenomena observed, although not established beyond doubt, is, however, so far in accord with the data thus far accumulated as to eliminate the necessity of assuming the activation of venom amboceptors bound to the corpuscles by true serum complements.

The exact modifications of cells which may occur in the above instance, as in the case of the indirect activators, are not known. Doubtless many substances will eventually be recognized which may modify the cell susceptibility by rendering its membrane more permeable, by extracting intracellular inhibiting substances, by modifying the arrangement of intracellular substances, or possibly by acting as catalyzers in the reaction of the lysin with the cellular constituent upon which it operates. In all attempts to analyze such modifying conditions, however, it is necessary to recognize distinctly the difference between the action of intracellular substances in accelerating the rate of hemolysis and in increasing the degree of hemolysis. The addition of certain constituents of serum and indeed of corpuscles themselves to a lysin and given corpuscles may markedly increase the rate of hemolysis without influencing however the degree of hemolysis ultimately to be observed. I emphasize the necessity of this distinction, inasmuch as it has been repeatedly overlooked by recent workers. Such readings, for instance, as those made after one and one-half, two, and four hours by von Dungern and Coca throughout their experiments are of but little value as quantitative data concerning the amount of hemotoxin actually present in a given instance. Such observations are of

value only as indicating the rate of hemolysis. The end reaction, which in most cases of venom hemolysis can be observed only from ten to twenty-four hours, is the only safe criterion as to the true hemolytic power of a given toxin. By the way of illustration the following experiment serves to show the influence of an aqueous extract of guinea-pig erythrocytes in strikingly increasing the rate of venom hemolysis without modifying the extent of such hemolysis (Table 55).

TABLE 55.

To each tube of one series (I) was added 1 c.c. of physiological salt solution and to each tube of the second series (II) was added 1 c.c. of the salt solution employed for the second washing of the corpuscles and representing aqueous extract of same.

Amount of Cobra Venom (0.02 per cent)	1 c.c. 5 per cent Suspension of Guinea-Pig Erythrocytes Hemolysis Observed After:			
	A. ½ Hour at 37° C.		B. 2 Hours at 37° C. and 16 Subsequent Hours at 10° C.	
c.c.	I) +1 c.c. Salt Solution	II) +1 c.c. Wash Water	I) +1 c.c. Salt Solution	II) +1 c.c. Wash Water
1.0	0	complete	complete	complete
0.75	0	"	"	"
0.5	0	"	"	"
0.35	0	"	"	"
0.25	0	"	*complete*	*complete*
0.15	0	marked	almost complete	almost complete
0.1	0	medium	medium	marked
0.075	0	faint trace	slight	slight
0.05	0	0 (?)	almost 0	faint trace
0.035	0	0	0	0
0.025	0	0	0	0
0.015	0	0	0	0

In the above experiment it is seen that conditions which may have no effect upon the ultimate hemolysis produced by cobra venom may nevertheless markedly increase the rate of hemolysis, and that any reading of the comparative degree of hemolysis prior to the end reaction will lead to a fallacy concerning the hemolytic power of the same venom under the two conditions.

In concluding this section concerning serum activation I wish to repeat the statement made earlier in the discussion that the data thus far obtained justify only provisional conclusions, and, further, to emphasize the fact, that although sera are known to modify venom hemolysis in several, if not many ways, there exists at present no proof of a true activation of venom hemotoxin by serum constituents other than lecithin.

VI.

GENERAL CONSIDERATIONS.

In the present section it is intended to discuss certain more or less isolated phases of venom hemolysis which do not fall logically under the headings of the previous sections.

The discovery of the activating action of lecithin for the venoms stimulated a wide appreciation of the important rôle of the lipoids in the field of immunity. Among other results, it has occurred that numerous investigators have attempted to effect, under dissimilar conditions, activations similar to that displayed by lecithin with the venoms. The lecithin reaction appears, however, to be one of not very general occurrence and unfortunately much confusion has arisen from attempts to draw close analogies where such analogies do not exist. Thus the results obtained by Pascucci[1] with lecithin and ricin, by Landsteiner and Jagic[2] with lecithin and silicic acid, by Reiss[3] with chloroform solutions of lecithin and trypsin ferment, by Iscovesco[4] with lecithin and colloidal iron; all, while adding excellent data concerning certain properties of lecithin, are not to be confused as examples of a true lecithin activation similar to that observed in the formation of venom lecithids. In these instances, no evidence of a chemical reaction is furnished by the isolation of a newly formed end product and indeed there is no indication whatsoever of a chemical reaction between the substances involved. The phenomena observed are referable in the individual instances either to a physical mixture of the reagents or to an independent action of the two reagents upon the cell indicator employed.

Also Michaelis and Rona[5] have failed to draw a distinction between certain physical mixtures and the chemical reaction which occurs in the formations of venom lecithids. These authors found that rennin is precipitated from its aqueous solution by mastix and that the precipitate thus obtained is soluble in chloroform. On the basis of this finding and the correspondence in chloroform solubility of mastix-rennin with that of the cobra lecithid, these authors have attempted

[1] *Beiträge z. chemisch. Phys. u. Path.*, 1906, 7, p. 457.

[2] *Wien. klin. Wchnschr.*, 1904, 17, p. 63.

[3] *Berl. klin. Wchnschr.*, 1904, 45, p. 1169.

[4] *Compt. rend. de la Soc. de Biol.*, 1907, 63, p. 744.

[5] *Biochem. Ztschr.*, 1907, 4, p. 11.

to establish a complete analogy between the mastix-rennin precipitation and the lecithin-venom reaction, reaching the conclusion that in the latter instance, as in the former, the essential reaction is that of a physical absorption by two colloids.

Now in section IV it has been shown that the end product of the reaction between lecithin and venom is, so far as can be determined by an elementary chemical analysis, composed largely of monostearyl-lecithin. It is at once necessary, therefore, in making any possible application of the hypothesis advanced by Michaelis and Rona, to assume that at first there actually does occur a chemical reaction in the splitting of monostearyl-lecithin from lecithin, and that secondly there occurs an absorption of the active hemotoxin constituent by this split product. The final question determining the validity of the Michaelis-Rona hypothesis would then be, whether or not the venom lecithid is a mixture of a split product of lecithin and native hemotoxin or is a single newly formed hemolysin.

The physical and chemical proofs that cobra lecithid is indeed a newly formed substance have been discussed in section IV but I may again point out in this connection the fact that the lecithid as a toxin displays distinctive characteristics which show that its lytic action cannot be referred to either of the substances employed in its production, as such. Among these characteristics are the following:

1. The extreme rapidity of its hemolytic action;
2. The absolute heat resistance, even to boiling;
3. The lack of susceptibility to the neutralizing action of antivenin, and
4. The possibility of producing with the lecithid a new antibody of greater affinity than that possessed by antivenin.

Furthermore, it is not possible to recover from the lecithid a trace of lecithin or of native hemotoxin; and again as shown in the following table, the hemolytic action of the lecithid is not increased either by the addition of unmodified venom or of free lecithin (Table 56).

Contrasting sharply with the characteristics of the lecithid which show it to be a newly formed chemical substance, are the properties of the mastix-rennin preparations of Michaelis and Rona. The details given by these authors show that unmodified rennin may be recovered from their preparations: Also the rennin as mixed with the mastix is seen to retain marked characteristics of native rennin among which is an identical susceptibility to destruction by heat.

The mastix-rennin preparations described by Michaelis and Rona do indeed give all evidence of being physical mixtures and such only. In this however they differ so essentially from cobra lecithid that analogies between these two classes of preparations are unwarranted and deductions therefrom unreliable.

TABLE 56.

INDIFFERENCE OF COBRA LECITHID TO PRESENCE OF LECITHIN OR OF NATIVE VENOM.

COBRA LECITHID 0.1 PER CENT c.c.	1 C.C. 5 PER CENT SUSPENSION OF GOAT ERYTHROCYTES		
	a) Cobra Lecithid Only	b) Cobra Lecithid + 0.1 c.c. 0.1 per cent Lecithin	c) Cobra Lecithid + 0.2 c.c. 0.1 per cent Unmodified Cobra Venom
1.0	complete	complete	complete
0.75	"	"	"
0.5	"	"	"
0.35	"	"	"
0.25	almost complete	almost complete	almost complete
0.15	medium	medium	medium
0.1	almost 0	almost 0	almost 0
0.075	0	0	0
0.05	0	0	0
0.035	0	0	0
0.025	0	0	0
0.015	0	0	0

In contrast to the contributions above cited in which the criteria of true lecithin activation have, it appears to me, been more or less confused stands the work of Morgenroth and Carpi[1] concerning a lecithin activation of bee poison. These authors have demonstrated a true lecithin activation of bee-poison hemotoxin by isolating a lecithid of that constituent and their results are conclusive in showing that the hemotoxin of this secretion is comparable in its action to the hemotoxins of the snake and scorpion venoms. Also this work corroborates the chemical nature of the reaction between native hemotoxins and lecithin in the elaboration of lecithids. The work of Friedemann[2] concerning lecithin activation of a hemolytic principle extracted from the pancreas displays certain indications of a true activation. Unfortunately, however, Friedemann has not as yet produced final proof by isolating a lecithid and his results are, therefore, less conclusive than those of Morgenroth and Carpi.

Confusion arising from forced analogies is also to be observed in certain phases of the work of Noguchi on venom hemolysis. In

[1] *Berl. klin. Wchnschr.*, 1906, 43, p. 1424.

[2] *Deut. med. Wchnschr.*, 1907, 33, p. 585.

a given instance for example,[1] this investigator, while admitting the activating action of lecithin in general, has sought to show that the intracellular lecithin of erythrocytes is not available for venom activation, and that the true endocomplement of susceptible corpuscles is a fatty acid.

In a previous section I have emphasized the distinction long since made, between the true activation by lecithin (including cephalin) and the pseudo-activation by the fatty acids, etc., pointing out that in the latter instance the rôle of the fatty acids is not that of reacting with the venom, but of rendering the intracellular lecithin available for its reaction with the venom. It is in confusing this indirect action of the fatty acids with true activation, that Noguchi has been led to assume that these substances are essential intracellular activators which unite with the venom to form the ultimate lysin.[2]

The fundamental difficulty in accepting an interpretation such as that advanced by Noguchi is that all proof is lacking to show that a single one of the fatty acids or its soap, when isolated, is capable under any conditions of effecting a true activation of a venom. Before serious consideration can be given any substance as the essential intracellular venom activator it must first be shown that this substance in some form actually possesses the power of reacting with the venom to form a hemolytic substance. This has not been shown in the case of the fatty acids and their soaps and indeed Noguchi himself in a more recent publication ceases to draw strict analogies between the action of these substances and the activation by lecithin.

The fact that it is possible to extract with ether certain fatty acids from susceptible corpuscles which are not likewise obtainable from non-susceptible corpuscles is in full accord with the conception of intracellular lecithin activation: In those corpuscles which are susceptible to venom by virtue of the relatively loose combination of lecithin with their stromata, it might well be expected that other lipoids present were also loosely bound and hence relatively susceptible to extraction. It may indeed be possible that the presence of certain fatty acids in the stromata may be among the factors which determine the availability of the intracellular lecithin for reaction with

[1] *Jour. Exper. Med.*, 1907, 9, p. 436.

[2] Arrhenius (*Biochem. Ztschr.*, 1908, 11, p. 161) also fails to make a sufficient distinction between the action of lecithin with cobra venom and that of sodium oleate.

the venom. It cannot be maintained however that those corpuscles, from which the fatty acids are not recovered, are non-susceptible because of the absence of an intracellular activator, since, as Goebel[1] has shown, these same corpuscles are highly susceptible when suspended in an isotonic sugar solution. These cells do of course contain lecithin. The non-susceptibility of corpuscles in a given instance is not determined by the absolute absence of an intracellular activator, for an activating substance is at all times present (lecithin). The susceptibility or non-susceptibility is determined rather by the *availability* of this substance within the cell as determined by its relation to other constituents of the cell, which relation as shown by Goebel's experiments may vary under differing conditions.

The inhibiting action of calcium chloride for stroma activation, which Noguchi points to as demonstrating that the intracellular activating substance is not lecithin but a fatty acid, is far too variable to serve as an absolute criterion of separation between lecithin and other substances. If it be granted that, in general, the activating action of free lecithin is not inhibited by calcium chloride and that the activating action of the endocomplement-containing stromata is so inhibited, this does not eliminate lecithin as the activating substance in the latter instance. If there is one point which is well established in regard to lecithin it is that this substance behaves in all its reactions very differently in its various protein compounds. The fact that the activating action of free lecithin or even of ovovitellin is not inhibited by calcium chloride affords no basis for a generalization as to what reaction may be displayed by other distinctly different protein-lecithin compounds such as those of the stromata.

In view of the facts: that all corpuscles contain an activating dose of lecithin; that under favorable conditions all corpuscles are dissolved by venom alone; that all such corpuscles contain lecithin, and that this substance is the one substance known to form a lysin with venom, the contention that the intracellular lecithin is the essential endocomplement appears to retain its force.

In contrast to the mass of corroborative evidence concerning the activating action of lecithin are the claims of Bang[2] and some brief

[1] *Compt. rend. de la Soc. de Biol.*, 1905, 58, p. 420. [2] *Biochem. Ztschr.*, 1908, 11, p. 521.

space may best be given to the contradiction and explanation of these claims.

Bang has attempted to show that lecithin itself has no activating action even for the snake venoms. According to this author the apparent activation produced by lecithin preparations is not in fact due to lecithin but to other substances admixed with lecithin and insoluble for the most part in methyl alcohol.[1] The ether-insoluble lecithid described by me, Bang considers a substance pre-existent in commercial lecithin preparations and therefore, according to him, is not formed by a reaction between lecithin and venom.

The materials employed by Bang were of unfortunate selection and to the unsatisfactory character of his so-called lecithin preparations are to be referred the unusual results and deductions which he advances. Throughout that section of his work which purports to be a consideration of the lecithids described by me, Bang has proceeded with a false assumption, namely, that the lecithin which I employed in preparing specimens of cobra lecithid for analysis was crude commercial lecithin such as he himself chose for description and experimentation. As a matter of fact the lecithin which was actually employed in the preparation of the complete cobra lecithids used for chemical analysis was isolated with great care. I refer to those specimens kindly put at my disposal by such authorities on lecithin isolation and structure as P. Bergell and W. Koch, and to specimens isolated by myself. These lecithins contained no substances insoluble either in ether or in alcohol and were, in all instances where the isolation of a pure end product was attempted, several times reprecipitated with acetone immediately prior to their use for the elimination of free fatty acids. When Bang affirms the presence of admixtures in certain crude preparations of commercial lecithin he is discussing facts long since established. When, however, he assumes the presence of such substances in the lecithin actually used by me in preparing lecithids for analysis and deduces therefrom that such lecithids could only be impure products, he is in error.

Further, Bang, in attempting the isolation of lecithin from egg yolk by ether extraction, found that such lecithin as he obtained had

[1] It should be recalled in this connection that certain of the lecithins (cephalin) are insoluble in alcohol (cf. W. Koch).

a relatively slight activating power, and from this concluded that lecithin is presumably not an activator for cobra venom. Here again it appears that Bang was at least unfortunate as to the materials which he isolated and tested, since invariably preparations of lecithin isolated from the same source by fully competent investigators shows a constant activating power for cobra venom (Koch-Bergell). In considering the failure of Bang to isolate an activating lecithin from egg yolk, it is to be noted (1) that Bang relied for his primary extraction entirely upon ether as a solvent, whereas it is well recognized that hot alcohol is the one efficient reagent for the complete extraction of lecithin under such conditions;[1] (2) that his extraction results, where quantitatively controlled, showed wide variations, and (3) that he omits all chemical tests to show that the isolated substances which he assumed to be lecithin were actually such or even in part such.

Had Bang sought by a less tedious and more trustworthy method to determine whether it is the lecithin itself of a given preparation which reacts with cobra venom to form the lecithid, he might have made a direct determination of the lecithin content of the preparation before and after its reaction with cobra venom in the formation of such a lecithid. He would have found that coincidently with the formation of the ether insoluble lecithid, there is a proportionate disappearance of the lecithin together with the appearance of free fatty acids split therefrom, so that with suitable proportions of venom approximately the entire lecithin content of the preparation is finally exhausted in the formation of the highly lytic end product—lecithid. In elimination of Bang's contentions other proofs of the actual participation of lecithin in a chemical reaction with venom are not wanting and for corroboration of the more obvious instances the reader is referred to the summary given elsewhere.[2]

Von Dungern and Coca,[3] while substantiating the occurrence of a chemical reaction between lecithin and the venom resulting in hemolytic lecithids, have attempted to show that the lecithids them-

[1] Cf. Hoppe-Seyler, *Handbuch*, 7th ed., p. 157; Kyes and Sachs, *op. cit.;* Noguchi, *op. cit.*

[2] Cf. von Dungern and Coca, *Biochem. Ztschr.*, 1908, 12, p. 407.

[3] *Münch. med. Wchnschr.*, 1908, 55, p. 437, and *Biochem. Ztschr.*, 1908, 12, p. 407.

selves are simple split products of lecithin and lack any possible venom constituent which would justify their recognition as toxins.

This claim of von Dungern and Coca is indicated, according to them, by the facts (1) that simple splitting of lecithin in the absence of venom yields hemolytic substances; (2) that the chemical analysis of cobra lecithid approximates that of monostearyl-lecithin, and (3) that the antibody secured in certain experiments performed by them was not an anti-lecithid but an antibody to native venom hemotoxin.

It is indeed surprising, in view of the now extensive literature concerning the hemolytic action of fats and fatty acids, that investigators should attach special significance to the fact that certain products of lecithin, resulting from the splitting of this substance in the absence of venom, should be hemolytic. Just so surely as fatty acid radicals are combined in the lecithin molecule, so surely will any splitting of lecithin freeing such fatty acids allow the isolation of hemolytic substances. But the question in this instance is not, whether lytic substances may be split from lecithin, as indeed they may, but rather whether any such substances possess all the properties displayed by the lecithids. As will be seen later, they do not.

The second point upon which von Dungern and Coca based their conclusions is one to which I drew attention in a previous publication and there discussed.[1] It is true that by present methods of chemical analysis no difference in structure can be detected between monostearyl-lecithin and the lecithids, but it is the universal experience of investigators that direct chemical analyses, as now practiced, are by a long way insufficient for the determination of even the grosser structural characteristics of the true toxins. It may be disappointing that a chemical analysis of the lecithids does not yield explicit data concerning the venom constituent of those compounds, but it is not surprising in view of the fact that chemical methods have not to the present succeeded in revealing the structural peculiarity of a single true toxin to which its specific physiological action may be referred. The molecular weight determination of cobra lecithid shows that the lecithid molecule is one of extreme size, in which many fatty acid radicals are linked together by the hemotoxin constituent, and in the presence of such a preponderance of fatty acid radicals it is not to be

[1] Cf. Kyes, *Biochem. Ztschr.*, 1907, 4, p. 109.

expected that the slight amount of the venom constituent would be susceptible to analysis or even recognition by present direct chemical procedures so notoriously inefficient for the analysis of the true toxins in general.

On the other hand, certain biological reactions, much more sensitive to structural differences in toxins, show clearly that a true toxin constituent exists in the lecithid compound. I refer to the extent and character of the hemotoxic action of the lecithids and to their power of stimulating the production of specific antibodies which effect not only their own neutralization but that of the native hemotoxin from which they are compounded. The elaboration of the latter point requires a comparison of the immunization to cobra lecithid given in section IV with that attempted by von Dungern and Coca, and cited by them as challenging the right of the lecithids to consideration as true toxins.

The comparison shows that whereas by immunization with a complete lecithid I obtained an antibody to that substance, von Dungern and Coca in their experiments failed to obtain such an antibody. The cause of this difference in results is not difficult to determine and is seen to exist chiefly in a difference in the materials used for immunization. The lecithid which I employed was in all instances a fully isolated complete lecithid free from admixtures of incomplete lecithids and native hemotoxin. Von Dungern and Coca on the other hand attempted the preparation of a lecithid by a method which I devised early in the venom work but which I was subsequently forced to modify because of its unreliability in producing a satisfactory yield of complete lecithid (cf. p. 217). Although the modified method was published in detail, von Dungern and Coca chose the older method, and with this method obtained preparations which as shown by their protocols were mixtures of incomplete lecithid, native hemotoxin, and a certain amount of complete lecithid. With such preparations these authors inoculated animals, and after a relatively brief period (twelve days), obtained a serum which, in contrast to that obtained by me, possessed a neutralizing action, not for both native venom and lecithid, but for native venom alone. It was from this finding von Dungern and Coca reached the conclusion that cobra lecithid, as such, does not stimulate the production of a

specific antibody and is not, therefore, a true toxin. Such experiments do not however warrant the generalization which von Dungern and Coca offer.

Considering the fact that the mixtures employed by von Dungern and Coca for immunization contained native hemotoxin, it is, of course, not surprising that these authors should obtain an antibody for this toxin, but such a result furnishes no logical basis for the deduction that a complete lecithid cannot be made to produce an antibody. Such results establish only this, that insufficient immunization with a mixture of incomplete lecithid, native venom, and a certain amount of complete lecithid, may produce at a given point an antibody to the native venom without coincidently producing an antibody for the complete lecithid. Had von Dungern and Coca prolonged the immunization sufficiently to have obtained antibodies for *all* of the toxins present in their mixtures, they doubtless would have found an amount of anti-lecithid in addition to the anti-native hemotoxin. But performed, as these experiments were, with ill-prepared material and extending in all throughout but twelve days, the results demonstrate only that under sufficiently unfavorable circumstances a lecithid may fail to produce its antibody.

Under more favorable conditions, however, as I have shown in section IV, sufficient immunization with a well-isolated complete lecithid results in the production of a specific anti-lecithid, and this fact in itself establishes the toxin nature of the lecithid. Furthermore, the anti-lecithid so obtained neutralizes also the hemotoxin of the native venom from which the lecithid was prepared, showing that the lecithid embraces a group derived from the native venom hemotoxin.

A phenomenon of especial interest which has been repeatedly observed in venom hemolysis is this, that maximum doses of a given venom may fail to produce hemolysis in instances where a smaller dose of the same venom produces typical and complete hemolysis.

Although the occurrence of this phenomenon is not in dispute, there exists no general concurrence of opinion as to its proper explanation.

Stephens,[1] in 1898, advanced the view that in such instances the

[1] Thesis, University of Cambridge, 1898; also *Jour. of Path. and Bact.*, 1900, 6, p. 273.

maximum doses of venom so modify the cell structurally as to inhibit lysis and the outflow of hemoglobin, and Noguchi[1] more recently has produced experimental data in support of this view. An explanation which I suggested in 1902[2] on the other hand was based on the quantitative relation between the intracellular lecithin and the venom. It was observed that the phenomenon under discussion occurred chiefly with those species of erythrocytes which contain relatively little available lecithin and also that within a given species the inhibiting action of maximum doses was marked in those individuals where the least intracellular lecithin was available. Furthermore it was shown that where certain susceptible corpuscles which had been treated with maximum doses of venom were removed from the venom and suspended in salt solution, these cells did not undergo hemolysis, but that when complements were added however, hemolysis was pronounced. (Cf. Table 57.)

TABLE 57.

	1 c.c. 5 per cent Suspension of Rabbit Erythrocytes + 1 c.c. 5 per cent Cobra Venom, 2 Hrs. at 37° C., Centrifugalized and Washed Erythrocytes +			Controls: Normal Rabbit Erythrocytes + 0.15 c.c. Guinea-Pig Serum or 0.5 c.c. Laked Guinea-Pig Erythrocytes
	a) 0.85 per cent NaCl Solution	*b*) 0.15 c.c. Guinea-Pig Serum	*c*) 0.5 c.c. Laked Guinea-Pig Erythrocytes ($\frac{1}{3}$)	
Resulting hemolysis	0	complete	complete	0

The result of the above experiment was taken to mean that after treatment of the corpuscles with the strong venom solution, there was insufficient endocomplement available within the cell to activate the hemotoxin amboceptors taken up by these cells but that upon the addition of a supplementary amount of extracellular complements the activation occurred with resulting hemolysis. In explaining the reduction of the amount of available endocomplement it was suggested that venom constituents which did not unite with the cell to produce hemolysis, but which possessed complementophile groups, appropriated a certain essential amount of the endocomplement.

Now, if in the case of susceptible corpuscles the lack of hemolysis in maximum doses of venom is indeed due to a relation between the

[1] *Jour. Exper. Med.*, 1905, 7, p. 1.

[2] *Berl. klin. Wchnschr.*, 1902, 39, p. 886.

intracellular lecithin and the excess of venom, the same reaction should appear when the stromata of susceptible corpuscles are employed in a fixed amount for the activation of the venom for non-susceptible corpuscles. In fact this is actually what occurs. Thus a minimum amount of endocomplement which produces lysis of the non-susceptible corpuscles with a moderate dose of venom fails to produce such hemolysis where a maximum amount of venom is employed. But further, if the lack of hemolysis by maximum doses of venom depends upon a relation between the venom and intra-cellular lecithin, a similar blocking might be expected when extra-cellular lecithin is employed. In other words, we should expect to find that small doses of extracellular lecithin just sufficient for the activation of moderate doses of venom for non-susceptible corpuscles would fail to produce hemolysis when the amount of venom was increased to the maximum. As a matter of fact, this is the result which obtains.

From these results it might also be expected that the added presence of a certain amount of extracellular lecithin to those corpuscles which contain a relatively small amount of available lecithin would eliminate the blocking of the hemolysis by maximum doses of venom. Such is actually the case. The addition of extracellular lecithin to the suspension of rabbits' corpuscles which are to be tested for a hemolysis blocking eliminates this phenomenon and the corpuscles are dissolved by maximum doses of venom as are those of guinea-pig and man.

The sum total of such results then may be taken as a strong indication that the blocking phenomenon is dependent upon quantitative relationship between the venom doses employed and the available lecithin.

On the other hand, results obtained by Noguchi do point directly to the fact that, in given instances at least, venoms which cannot alone dissolve the corpuscles increase the resistance of cells to certain hemolytic agents. Whether or not the constituents of the venom which effect this modification actually play a rôle in the phenomenon under discussion, is yet to be shown, for Noguchi in his experiments unfortunately did not employ a venom which even in medium doses

caused hemolysis of the corpuscles used. It must not be overlooked that the phenomenon to be analyzed is the failure of maximum doses to hemolyze under conditions where lesser doses do hemolyze.

Sachs[1] has also pointed out the fact that the great variation in amount of the same venom required to effect the inhibition with different individuals of the same species speaks against a simple fixing reaction on the part of the venom. Thus, where the blocking of hemolysis by 0.1 c.c. of a 1 per cent solution of cobra venom occurs with one specimen of rabbit's blood, 10 times this amount is required to produce the same blocking under the same conditions but with corpuscles from another individual. Furthermore a general "fixing" action on the part of venoms cannot be assumed for erythrocytes in general since the great majority of the corpuscles show a susceptibility to maximum as to medium doses of venoms.

Noguchi attaches undue importance, it seems to me, to those experiments in which by heating a venom he removes its blocking action without at the same time destroying its hemolytic action. This result means, according to Noguchi, that the hemotoxin is not the blocking constituent of the venom and further that the substance so destroyed acts only as a fixing reagent. In the first place in multiple repetition of Noguchi's experiments I have been unable to completely remove the blocking power of a venom without producing some diminution of its hemolytic action, and a destruction of hemotoxin, but slightly apparent, might of course well be sufficient to eliminate its deviating action. The exact quantitative results here are difficult to determine. On the other hand assuming that the substance which effects the blocking is other than the hemotoxin, it may well be that its action is that of deviating lecithin. It is by no means established that the active hemotoxin is the only constituent of venom with a lecithinophile group. It might also be considered possible that in heating the venom a certain amount of lecithin is liberated from the proteins of the venom itself, thus increasing the amount of complement. This however, I doubt to be the case.

Altogether, the blocking phenomenon appears as one of extreme complexity and is among those phases of venom hemolysis which require more quantitative experimentation before ultimate conclusions may be drawn.

[1] *Biochem. Centralbl.*, 1906, 5, p. 257.

The true toxin nature of the snake venoms, as indicated by the production of their antitoxins, was established relatively early, and these secretions have from the first played an extensive rôle in experimentation concerning the fundamental problems of immunity. Especially is this true concerning the analysis of the exact nature of the toxin-antitoxin reaction. Because of its adaptability to quantitative experiments *in vitro*, the hemolytic constituent of the venoms has been most extensively employed in this connection.

Myers[1] was the first to study exhaustively the quantitative relations between cobra venom hemotoxin and its antibody. Employing the partial neutralization method of Ehrlich, this investigator obtained results which, like the more recent analogous experiments of Flexner and Noguchi, indicated that this toxin is either highly complex after the nature of diphtheria toxin or that, being simpler, the affinity between it and its antitoxin is slight. The neutralization line as plotted approximated the parabolic curves observed in the fractional neutralization of a weak acid with a weak base (boracic acid and ammonia). It was indeed in this contribution that the first suggestion was made that irregularities in the toxin-antitoxin reaction might be referred to a dissociation of the toxin-antitoxin complex, and much use has been made of these experiments by Arrhenius and his followers who have since elaborated this theme *in extenso*.

The experiments of Myers were performed with care and were well controlled. With the development of new methods and the acquisition of new data concerning the mechanism of venom hemolysis, however, there arose distinct indications for the repetition of such experiments under somewhat modified conditions.

Thus the investigations concerning lecithin as an activator brought to light the fact that an accurate determination of the amount of hemotoxin in a given venom can only be obtained in the presence of an excess of extracellular complement. This condition was not satisfied in the experiments above referred to.

To determine more accurately, then, the fractions of hemotoxin neutralized by successive units of anti-hemotoxin, experiments were performed as follows (Table 58):

A given amount of cobra venom was added to various amounts of Calmette's antivenin and after a time the hemolytic action of each mixture was determined for

[1] *Jour. Path. and Bact.*, 1900, 6, p. 415.

ox corpuscles (non-susceptible) in the presence of a multiple activating dose of lecithin. Such an experiment is the following:

Four mixtures were thus prepared:

I.	1.5 c.c. 1 per cent cobra venom		+13.5 c.c. salt solution
II.	1.5 " " " "	+0.75 c.c. antivenin	+12.75 " " "
III.	1.5 " " " "	+1.5 " "	+12 " " "
IV.	1.5 " " " "	+2.25 " "	+11.25 " " "

Each of these mixtures was allowed to stand 15 hours at room temperature, after which various dilutions of each mixture were tested for hemolytic action upon 1 c.c. of a 5 per cent suspension of ox erythrocytes in the presence of 0.2 c.c. of a 0.1 per cent solution of lecithin. The amount of hemolysis produced by each mixture is shown in the following table:

TABLE 58.

Amount from Each Diluted Mixture c.c.	Mixture I Diluted 1/300	Mixture II Diluted 1/200	Mixture III Diluted 1/100	Mixture IV Undiluted
1.0	complete	complete	complete	slight
0.97	"	"	"	"
0.95	"	"	"	"
0.92	"	"	"	"
0.9	almost complete	almost complete	almost complete	trace
0.87	" "	" "	" "	"
0.85	" "	" "	" "	"

The computation of the hemolytic doses contained in each of the four mixtures of 15 c.c. gives the following results:

TABLE 59.

Mixtures	Number of Lytic Doses Remaining	Decrease in Number of Lytic Doses
I. (Toxin alone)	4,890	....
II. (Toxin+0.75 c.c. serum)	3,260	1,630
III. (Toxin+1.5 c.c. serum)	1,630	1,630
IV. (Toxin+2.25 c.c. serum)	5 (approximately)*	1,625

* Three c.c. of mixture caused complete hemolysis.

From this experiment it appears that each 0.75 c.c. of antivenin neutralized the same amount of venom hemotoxin (1,630 lytic doses). Accurately determined then on the basis of the actual maximum of unneutralized hemotoxin in the respective mixtures, the neutralization of cobra hemotoxin by its antibody corresponds in type to the neutralization of a strong acid with a strong base. In other words, the plot of the partial neutralization displays a straight line and not the parabolic curve obtained with weak alkalies and weak acids. This result, many times paralleled, is of especial interest and its

significance will be discussed at a later point. It shows that the cobra hemotoxin is a simple toxin of marked affinity for its antitoxin.

It has been suggested above, that the results obtained by Myers and by Flexner and Noguchi which do not correspond to those just given were due to a lack of sufficient activating substances in the hemolytic experiments to insure an accurate determination of the amount of unneutralized hemotoxin in the several toxin-antitoxin mixtures. Were this actually the case, a sufficient decrease in the amount of lecithin used in experiments such as the one just given should produce results approximating the findings of these authors. This is indeed the case as shown by the following experiment in which a single activating dose of lecithin was substituted for the excess of lecithin in the above experiment.

TABLE 60.

Mixtures as in Previous Experiment Contain 1.5 c.c. 1 per cent Cobra Venom	Number of Lytic Doses Remaining	Decrease in Number of Lytic Doses
I. (Toxin alone)	2,250	...
II. (Toxin+0.75 c.c. serum)	1,380	870
III. (Toxin+1.5 c.c. serum)	645	735
IV. (Toxin+2.25 c.c. serum)	0	645

It is to be noticed in the above table that, so far as indicated by the hemolysis, the amount of toxin neutralized by the increasing doses of antitoxin did not appear directly proportionate to the amount of antitoxin added. The results obtained thus are comparable to those of Myers. The error in accepting such results as data concerning toxin neutralization is that the degree of hemolysis displayed by the given mixtures under the conditions imposed, is not a true index of the amount of unneutralized hemotoxin actually contained in such mixtures.

Why a minimum activating dose of lecithin is insufficient to guarantee a full expression of the amount of uncombined hemotoxin present in experiments such as the above, appears to be explained by the power of the toxin-antitoxin complex to deviate a portion of the lecithin, thus leaving an insufficient amount free for the full activation of the unneutralized hemotoxin.

The basis for such an explanation is to be seen in the following experiment:

The minimal lytic dose of each of the following three mixtures of venom and antivenin was determined in the presence of an excess of lecithin.

I.	0.5 c.c. 1 per cent cobra venom	+4.5 c.c. salt solution			
II.	0.5 " " " "	+0.5 "	antivenin	+4	c.c. salt solution
III.	0.5 " " " "	+1.0 "	"	+3.5	" salt solution

Next, $1\frac{1}{2}$ the lytic dose, as thus determined, was added to each of the series of tubes containing 1 c.c. of a 5 per cent suspension of ox corpuscles. With these tubes as indicators the amount of lecithin necessary to effect complete hemolysis was determined in each of three instances. Inasmuch as the amount of unneutralized hemotoxin in all three series was presumably the same ($1\frac{1}{2}$ lytic doses), it might have been expected that the amount of lecithin required for activation would, therefore, be the same. Actually, however, as shown in the following table, a greater amount of lecithin was necessary where the greater amount of toxin-antitoxin combination was present.

Inasmuch as horse serum itself unheated as well as heated activates rather than inhibits venom hemotoxin, cholesterin inhibition by the antitoxin (horse) serum added can be eliminated in the above experiment, and it appears clearly that the hemotoxin-antitoxin complex modifies the hemolytic action of the uncombined hemotoxin by deviating an essential amount of the lecithin hemotoxin complement. In this inhibition of hemolysis by the deviation of the lecithin is to be recognized a process analogous to that which I have previously outlined in explanation of the blocking phenomenon displayed by maximum doses of venom. A similar instance has been observed also by Morgenroth[1] in the case of serum lysins, where the serum amboceptors, neutralized by the anti-amboceptor and present in excess, deviate the complement.

The power of the toxin-antitoxin complex to bind lecithin is also demonstrated in the procedure given below.

It is to be remembered that the venom hemotoxin, when shaken with a chloroform lecithin solution, disappears from its aqueous solution appearing in the chloroform solution from which it is isolated as a lecithid by ether precipitation. Now, when a neutralized mixture of venom hemotoxin and antivenin is shaken with chloroform lecithin, no precipitate appears in the chloroform solution when ether is added. In other words, there is no indication of a transfer of the hemotoxin complex from the aqueous to chloroform solution. It might be assumed, however, that the shaking of such a neutral mixture with chloroform lecithin results in the transfer of a certain

[1] *Centralbl. f. Bakt.*, 1904, 35, p. 501.

amount of lecithin to the aqueous solution to form a lecithin-hemotoxin-antivenin compound. It should then be possible to recover lecithin from such a compound by the alcohol precipitation of the aqueous solution. Such is actually the case, as shown by the following detailed experiment (Table 61):

To 0.5 c.c. of a 1 per cent. cobra venom solution 1 c.c. of an antivenin was added; the whole being diluted to 5 c.c. and allowed to stand at room temperature for 15 hours (solution A). Two c.c. of solution A was then shaken with a chloroform lecithin solution, 1.5 c.c. of the aqueous portion was then recovered, and just the amount of native cobra venom added to make the hemolytic power of this solution (solution B) with an excess of lecithin exactly equal to the hemolytic power of solution A. Solutions A and B were then precipitated with 9 volumes of alcohol and the respective precipitate was redissolved in equal amounts of physiological salt solution. Finally the amount of lecithin necessary for the activation of the lytic dose of these solutions was determined.

TABLE 61.

Amount of 0.01 per cent Lecithin Solution c.c.	1 c.c. 5 per cent Suspension Ox Erythrocytes + Single Lytic Dose of:	
	A	B
0.57	complete	complete
0.5	almost complete	"
0.45	marked	"
0.4	"	"
0.35	medium	"
0.32	slight	almost complete
0.32	"	marked

The above table shows that solution B, which had previously been treated with chloroform lecithin, lacked the lecithin-deviating power displayed by solution A, which contained the lecithin-free toxin-antitoxin complex.

In the total, the experiments cited give adequate proof that in order to obtain a true estimate of the amount of uncombined hemotoxin in partially neutralized venom hemotoxin mixtures, sufficient extracellular complement must be added to eliminate the complicating factor of complement deviation by the antitoxin-toxin complex; and that when an excess of lecithin is employed to this end, the correct determination of the amount of toxin neutralized by each added amount of antitoxin plots in a straight line and not a parabolic curve.

The general significance of this fact is that in the instance of the venom hemotoxin-antitoxin reaction there is no indication that the reaction is reversible after the manner of the reaction between weak

acids and alkalies and such support as Arrhenius and his followers have drawn from the earlier neutralization experiments with the venoms rests upon what I have shown to be errors in experimentation. The same experiments conducted with the elimination of these errors not only fail to furnish proof for the view which holds that all irregularities appearing in toxin-antitoxin neutralizations are to be explained on the basis of the dissociation of toxin-antitoxin complex, but rather show that in an instance where toxoids and toxons are lacking, the irregularities in neutralization are also lacking and the neutralization follows the law of multiple proportions.

In concluding I wish to construct only a most general summary, recognizing that in this, the one instance where a true toxin is placed tangential to structural chemistry, the major value of the work is most surely the general point of view which it establishes, and in the more or less detached details of experimentation which may find broader application in other investigations. In such a summary the following points may be emphasized:

1. That there is present in all venoms a hemolysin, existing as one of a number of distinct toxins.

2. That this hemotoxin effects hemolysis only in conjunction with a so-called complementing substance which however may be found within the erythrocytes.

3. That so far as at present recognized the activating substances are lecithins.

4. That the reaction between the hemotoxin and lecithin is essentially a chemical reaction resulting in the formation of a complete lysin.

5. That this complete lysin is a true toxin in that it stimulates the production of a specific antitoxin.

STUDIES IN THE MORPHOLOGY OF MALARIAL PLASMODIA AFTER THE ADMINISTRATION OF QUININE, AND IN INTRACORPUSCULAR CONJUGATION.*†

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In reviewing the literature upon the action of quinine on the malarial plasmodia, one is surprised to find how much confusion apparently exists as to the exact effect of this drug on the parasites, and, therefore, the most effective period for its administration in the treatment of the malarial fevers. Indeed, we find so great an authority as Manson[1] saying, in the latest edition of his work upon tropical diseases:

> In what way quinine acts has not been satisfactorily explained. Some, reasoning from the toxic influence this drug exerts upon many kinds of free amoebae, say that it acts in malaria in the same way; that is, as a direct poison to the parasite. . . . . Others maintain that it acts in stimulating the phagocytes, the natural enemies of the parasites. Some experimentalists allege, on the other hand, that it paralyses the white corpuscles. It is said by some to be most effective against the free spores and the very young intracorpuscular forms, but inoperative against the more mature parasites; hence they advocate giving it early in the parasitic cycle. Others, on the contrary, maintain that it is operative only on the large intracorpuscular forms, and therefore advocate its use at a late stage of the cycle.

This statement, coming from so distinguished an authority upon malaria, well illustrates the uncertainty that exists regarding the action of quinine upon the malarial plasmodia, despite the fact that the drug is known to be a specific in malarial disease, and that it is comparatively easy to determine the exact morphological changes produced by it in the plasmodia.

I have devoted much study to this subject and have examined many cases of malaria after the administration of quinine with a view to determining the exact morphological changes produced by

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† With permission of the Surgeon General, U. S. Army.

[1] *Tropical Diseases*, 4th ed., New York, 1908.

the drug in the various species of plasmodia, and the present contribution gives the results of this study, together with data concerning the process of intracorpuscular conjugation which I have collected since I described this phenomenon in December, 1905. The material for these studies has embraced infections with all of the species of human plasmodia, the infections being in the persons of soldiers of the army stationed in the Philippines and returning therefrom, as well as in native Filipinos. My method has been to examine the blood at regular intervals after the administration of quinine, studying both fresh and stained preparations, and in this way to observe the effect of the drug upon every stage of the life-cycle of the plasmodia in man; in some instances the drug was given in divided doses at regular intervals, while in others it was administered just before the expected paroxysm or at various stages of the development of the plasmodia. In every instance it was found that more pronounced results were obtained when the quinine was administered in divided doses, at regular intervals.

The material embraced in the study of intracorpuscular conjugation comprises many hundred cases of malarial infection, including infections with every species of human plasmodium, and the results are such that I am firmly of the opinion that this process is of great importance in the life-history of the plasmodia and that it explains most logically the occurrence of latent infection and recurrence in malarial disease. Certain it is, that the process is too frequently observed to be considered as accidental and that it is entirely distinct from the double and triple infection of corpuscles frequently observed, especially in estivo-autumnal infections.

The confusion existing regarding the action of quinine upon the plasmodia is, no doubt, largely due to imperfect staining methods employed by various investigators, and to the fact that more attention has been paid to the appearance of the parasites in the fresh blood than to their staining reactions. In my work I have employed the staining method of Wright, modified slightly as described in previous contributions.[1] I have found this method very satisfactory, as it is easy of application and uniform in its results. By it, the chromatin of the nucleus of the parasite stains a brilliant red, the vesicular

[1] Craig, *Amer. Med.*, 1905, 10, pp. 982, 1029; *Jour. Infect. Dis.*, 1908 5, p. 324.

portion of the nucleus remains unstained, while the protoplasm stains a robin's-egg blue.

Part I.

THE CHANGES IN THE MORPHOLOGY OF THE MALARIAL PLASMODIA PRODUCED BY QUININE.

In considering the morphological changes produced in the malarial plasmodia by quinine, it is necessary to describe those occurring in the forms concerned in schizogony, and also those occurring in the forms of the plasmodia developed in man which are concerned in sporogony. But before giving my results a brief summary of the results of the work of other observers may be of interest:

In 1867, Binz[1] found that solutions of quinine, when brought in contact with some of the infusoria, produce morphological changes ending in the death of the organisms experimented with, and from this fact drew the conclusion that the beneficial action of the drug in malarial infections was due to the destruction of the plasmodia. In 1881, Laveran[2] demonstrated that a 1:1000 solution of quinine produced immediate cessation of the movements of the plasmodia when it was added to blood containing them, and his results were soon confirmed by other observers.

As regards Plasmodium vivax (the tertian plasmodium), Golgi[3] claimed that quinine produced very decided effects, even the adult plasmodia being markedly changed in appearance. This work was done upon fresh blood specimens and he found that the drug produced a shrinkage in the parasites and a granular degeneration of the protoplasm, while the segmenting bodies were found to have a smaller number of segments. Romanowsky,[4] using his method of staining, found that in the young parasites the vesicular portion of the nucleus disappeared, and that the chromatin stained less intensely. In the older parasites he found that the protoplasm stained poorly; that the chromatin was reduced in amount and stained less intensely; and that the vesicular portion of the nucleus disappeared. Mannaberg[5] studied both fresh and stained specimens of the tertian plasmodium, finding that quinine produced in the former lessened ameboid motion and that several hours after the administration of the drug the parasites underwent fragmentation. He claimed that in stained preparations the chromatin entirely lost its power of staining, and that most of the segmenting bodies were devoid of chromatin. On the other hand, Ziemann found that when the drug is given during apyrexia the chromatin stained as usual, altho fragmentation of the parasites occurred.

The later observations of Schaudinn[6] are of interest in that he studied preparations stained by the Romanowsky method, in which the plasmodia were present in every

[1] *Archiv f. path. Anat.*, 1867, 41, p. 23.

[2] *Compt rend. Acad. Sci.*, 93, p 627.

[3] *Deut. med. Wchnschr.*, 1892, 18, pp. 613, 685, 707. 729.

[4] *St. Petersburg med. Wchnschr.*, 1891, Nos. 34, 35.

[5] *Die Malarial Krankheiten*, Nothnagle, Philadelphia, 1905.

[6] *Arb. a. d. kais. Gesundh.*, 1902, 19, p. 169.

stage of their human life-cycle. Unfortunately he studied only one case of double tertian infection, so far as can be judged by his paper, and it is therefore hardly justifiable to consider his results as conclusive for quartan or estivo-autumnal infections, or even for tertian infections as a class. Schaudinn found that quinine rendered the young tertian schizont less stainable and deformed in appearance; the nucleus, as well as the protoplasm, was fragmented, altho the nuclear chromatin still stained. In the older schizonts fragmentation of both the protoplasm and nucleus was very common, while both stained poorly. He found no certain evidences of the action of the drug upon the reproductive phases of the plasmodia, except that the merozoites stained diffusely and the nucleus appeared broken and scattered. He found that quinine affected the developing sexual forms in the same manner as the schizonts, but that the fully developed gametes were resistant to its action.

As regards Plasmodium malariae (the quartan plasmodium), the observations are not as numerous and are conflicting. Antolisei[1] could not find any morphological changes in this plasmodium as the result of quinine, and his results were confirmed by Golgi, who claimed that the drug had no effect upon the adult quartan plasmodium. Mannaberg found the same changes occurring in this organism after quinine administration as were described by him in the tertian parasite.

The observations concerning the action of quinine upon the estivo-autumnal plasmodia (Plasmodium falciparum and Plasmodium falciparum quotidianum) are still more conflicting. Baccelli[2] found that the administration of the drug, while producing an increase in the ameboid activity, did not produce any degenerative changes; Marchiafava and Bignami[3] claimed that the drug caused the young parasites to leave the infected cells but that no morphological changes occurred, with the exception that pigment formation was hindered, thus proving that the drug interfered with the nutrition of the organism. La Monaco and Panichi[4] state that the effect of the drug varies with the strength of the solution employed; weak solutions causing swelling and increased ameboid activity; strong solutions greatly increasing ameboid activity; and that very strong solutions caused shrinkage and death. Their work was done upon fresh specimens, the quinine solution being added to the blood after it had been removed from the body.

From this brief summary it is evident that the results of various observers are conflicting and the reason for this is not always easily apparent. As regards the method of experimentation by adding quinine solutions to blood containing the parasites after the latter have been removed from the body, it is obvious that it is an imperfect one and one from which conclusions should not be drawn as regards the effect of the drug when it is circulating in the blood. Cappogrossi[5] has shown that the exit of the plasmodia from the red corpuscles, so often observed when a solution of quinine is added to

[1] *Riforma Med.*, 1890, 6, p. 68.

[2] *Studien über Malaria*, Berlin, 1895.

[3] "Malaria," *20th Century Practice*, New York, 1900.

[4] *Untersuchungen zur Naturlehre des Menschen und der Tiere*, 1901, 17, p. 22.

[5] *Il Policlinico*, 1901.

infected blood, is not due to the specific action of the drug, but is purely physical, being due to the hypotonicity of the solution. He found that an isotonic salt solution containing quinine does not cause the extrusion of the parasites from the red corpuscles but reduces their motility and renders their protoplasm more granular.

In detailing the results of my studies upon this subject I shall describe the changes produced by quinine upon all of the species of plasmodia occurring in man, as shown in both fresh and stained preparations of the blood, considering each species separately.

Plasmodium vivax (tertian plasmodium).—This species is most easily studied because of its comparatively large size and the ease with which the details of its morphology may be recognized. The changes produced by quinine in this species are very marked and are evident in the living organism as well as in stained preparations.

Fresh preparations.—Quinine produces marked morphological changes in this organism during every stage of its life-cycle in man, with the possible exception of that stage just preceding sporulation and the fully developed gametes. The action is most marked upon the young, rapidly growing plasmodia and least upon the fully developed forms. In the growing forms a great reduction is observed in the amount of pigment which is developed, thus proving that the drug influences very markedly the nutrition of the parasite. This is in accord with the observations of Marchiafava and Bignami. If quinine be administered at the time of sporulation it undoubtedly kills many of the merozoites at once, but many escape and undergo development, to a greater or less extent, within the red corpuscles. Death may occur at any stage of this development and the changes produced in the morphology of the parasite do not differ from those usually observed when the drug is given after the organisms have entered the erythrocytes. Those parasites, however, which survive and sporulate, generally present atypical sporulating forms, thus proving that the drug has influenced their development, even tho it has not destroyed them.

If the drug be administered, in a single tertian infection, at the time that the blood contains the young intracorpuscular plasmodia, the first change observed in the living parasite is a great increase in the ameboid activity, followed, after an hour or so, by a marked decrease. The ameboid activity is often so greatly stimulated that it is extremely difficult to follow the movements of the pseudopodia of the organisms, which are projected and withdrawn so rapidly as to make it almost impossible to keep them in focus. The "ring-form," so frequently observed in this type of infection, is lost during the period of stimulation, but as the motility becomes lessened it is regained, and after the cessation of motion the parasite is generally spherical or "ring-shaped," and more sharply outlined than is the normal organism.

The protoplasm becomes more granular in appearance, and while, in a case untreated with quinine, in which the parasites are in a similar developmental stage, a few granules of pigment are usually observed, pigment is never found in the plasmodia presenting the phenomena I have just described.

If the drug be continued and the blood be examined at regular intervals, it will be noticed that it acts upon the plasmodia in every stage of development up to the time of sporulation. Many of the organisms perish, for it is noticed that the number present in the blood specimens gradually decreases as the time for sporulation approaches, but some of the parasites mature and sporulate, altho nearly always presenting marked morphological changes, degenerative in character.

In the early pigmented stage of the parasite quinine produces the same initial stimulation of ameboid activity, followed, in many instances, by complete cessation of motion, and eventually, by fragmentation. After the formation of pigment quinine appears to produce fragmentation more frequently than while the parasite is in the hyaline stage of growth, and fragmented parasites are very numerous in the blood from tertian infections, if the blood be examined upon the fever-free day. The increase in the refractive index and the granular appearance of the protoplasm of the pigmented parasites is especially noticeable in those organisms which do not fragment but continue their development.

Examined during the afebrile period, the blood will be found to contain numerous erythrocytes which present within them plasmodia which are divided into several distinct portions, devoid of motion. These are fragmented parasites and should be carefully distinguished from those parasites in which ameboid motility is so pronounced as to cause the extrusion of numerous pseudopodia simultaneously, and their appearance at various portions of the infected cell. Many of the fragments contain pigment, which may be sluggishly motile. Sometimes the fragmented organisms appear to be extruded from the red corpuscle and may be observed lying free between the blood cells. The protoplasm of the fragments is generally more refractive than that of the normal plasmodium and much more granular in appearance. After liberation from the erythrocyte the fragments soon become very granular and finally disintegrate. It is very common in tertian infections, under treatment with quinine, to observe free plasmodia in almost every stage of development, presenting the degenerative changes mentioned. Such plasmodia are always more or less fragmented, the fragments frequently being held together by a delicate filament of protoplasm. The pigment within the fragments may remain motile for hours if the specimen of blood be kept upon a warm stage, but eventually the fragments separate, become mere masses of granular material, and disintegrate. The fragmentation due to quinine occurs at every stage of the human life-cycle of the tertian plasmodium up to the presegmenting stage, but at this stage and in the sporulating stage I have never observed any evidence of this form of degeneration.

While fragmentation terminates the career of many of the plasmodia which have escaped the action of quinine during the earlier period of development, some of the plasmodia continue to develop and go on to sporulation. Upon such parasites, up to the presegmenting stage, quinine, if given in one large dose, has a marked effect, causing shrinkage and granular deposits in the protoplasm; an increased refractive index of this substance; and a decrease in the amount of pigment developed during further growth. Some of the parasites are killed but the majority sporulate. It is rare to observe fragmentation in the more fully developed plasmodia, and it is very seldom observed in organisms which entirely fill the red corpuscles.

If the quinine has acted upon the plasmodia during their developmental cycle, as it does when it is administered in divided doses, the effects produced are similar in kind to those just described, but a smaller number of the parasites are able to sporulate,

having been so injured during their earlier stages as to perish in large numbers as sporulation approaches. Many of the organisms which do sporulate show a lessened number of merozoites and many of these are found to be sterile when stained specimens are examined.

So far as I have been able to determine, quinine produces no morphological changes in the presegmenting or segmenting parasites when it is administered at the time these stages are present in the blood. While this is so, it is evident that it destroys many of the merozoites after sporulation has occurred, as shown by the small number of these which can be demonstrated in the erythrocytes some hours after sporulation.

The morphological changes observed in living specimens of Plasmodium vivax, which are produced by quinine, may be summed up as follows:

a) An initial stimulation of ameboid activity, followed by a decrease in activity, and eventually in cessation of motion.

b) A granular degeneration of the protoplasm.

c) An increased refractive index of the protoplasm.

d) Fragmentation of the parasite, followed by the apparent extrusion of the fragmented organism from the red corpuscle.

e) A marked decrease in the amount of pigment developed in those parasites which undergo development during the administration of the drug. I believe that the extrusion of the fragmented parasites is only apparent, the real explanation of the process being that they are liberated by the death and disintegration of the infected erythrocyte. I have never observed the active extrusion of a malarial plasmodium from the red corpuscle and do not believe that it occurs as the result of the action of quinine.

The changes produced in the gametes are similar to those described in the forms of the human life-cycle but the drug affects the gametes only in the early stages of their development. The fully developed tertian gamete shows no morphological changes after the administration of quinine and I have repeatedly observed flagellation after the drug has been administered for several days. If, however, the quinine be administered early in the infection, the number of gametes is greatly reduced, many of them being destroyed during the early stages of their development.

Stained preparations.—While the morphological changes produced by quinine are easily observed in living tertian plasmodia, they are much better demonstrated in specimens stained with Wright's method, as in such preparations the chromatin of the nucleus is rendered visible and we are thus able to trace the action of the drug upon this essential portion of the parasite. My observations are confirmatory of those of

Romanowsky and Schaudinn in some particulars but I have been unable to demonstrate that the chromatin loses its staining power, as claimed by Romanowsky, or that it stains less intensely, as claimed by Schaudinn. I have found, on the contrary, that the chromatin stains more intensely after the administration of quinine than before, but that the quality of the staining is markedly affected. The brilliant red stain of the chromatin observed in normal specimens of plasmodia is replaced by a very dark purple or almost black color, indicating that some important change has occurred in the chemical nature of this substance. The unstained vesicular portion of the nucleus is found to have disappeared, and the chromatin is often arranged irregularly in the protoplasm in the young plasmodia, thus indicating that the nucleus has been broken up and the chromatin liberated. The claim made by Ziemann that the chromatin stains normally and does not become fragmented at any stage of the development of the parasite I have not been able to confirm. If the drug be given just before sporulation no change in the appearance of the chromatin is noticed but if the drug be administered early in the intracorpuscular stage of development, the markedly atypical division of the chromatin is very noticeable. The plasmodia which develop into sporulating bodies after the administration of quinine almost invariably present atypical conditions in stained preparations, the merozoites staining irregularly and being reduced in number, while many of them are devoid of chromatin.

The changes produced by quinine are best studied in stained preparations from patients to whom the drug has been given at three-hour intervals, the blood being examined at intervals of three or four hours. In this way it is not difficult to study the effect of the drug upon every stage of development of the tertian plasmodium as observed in the blood of man. The administration of the drug in a single large dose produces similar morphological changes, tho these are not so pronounced in character.

The effect of quinine upon the unpigmented schizonts, while still in the "ring-stage," is evidenced in stained preparations by the deeper staining of the protoplasm, the very dark violet or almost black staining of the chromatin, and the absence of the unstained area which normally surrounds the latter. The protoplasm takes a deep blue color, this substance in the normal plasmodium taking a lighter blue stain. The brilliant crimson color of the chromatin normally observed is replaced by a dark violet or almost black color, this change in staining reaction being always present in the young schizonts if the drug be administered just before or at the time of sporulation of the plasmodia. I have never observed fragmentation of either the protoplasm or nucleus at this stage of development, but minute irregularities in the contour of the "rings" are not infrequently noted, and very rarely the chromatin presents slight evidences of fragmentation, minute granules being present about the periphery of the large chromatin mass.

In organisms a little farther advanced in development the changes produced by the drug are similar to those just described but the stimulation of ameboid motion is shown in the very bizarre shape of the parasites. Fragmentation is not infrequently observed even in the young schizont after it has lost its "ring-form" and before the development of pigment. In such instances the infected erythrocyte contains two or more irregular masses of protoplasm stained a deep blue color while the chromatin may or may not be fragmented at this early stage. If the chromatin be fragmented it may be observed distributed in the fragments of protoplasm but where fragmentation has not occurred it is either contained in one of the detached portions of protoplasm or lies free within the erythrocyte. Rarely the extrusion of the chromatin is observed at this

stage of development. The chromatin takes a deep violet or almost black color. In most of the plasmodia the vesicular portion of the nucleus cannot be demonstrated.

The effect of the drug is most marked after the development of pigment and when the plasmodium is from one-half to three-quarters grown. In such plasmodia the evidence of fragmentation is most prominent and the changes in the chromatin most easily demonstrated.

Many of the erythrocytes contain fragmented plasmodia, the fragments being irregular in shape and staining a deep blue or almost purple color. Pigment may be present in them but the decrease in the amount of the latter as compared to the amount in normal plasmodia at the same stage of development is most striking.

The chromatin stains a dark violet or almost black color and there is little evidence of increase in the amount or of division of this substance. While in normal plasmodia at similar stages of development the chromatin has increased notably in amount and has divided into fine granules and filaments arranged loosely in one portion of the organism, in the plasmodia affected by quinine the chromatin has increased slightly, if at all, in amount, and is arranged in either one large clump or in two or three smaller ones; it is never observed in the form of small granules or threads in parasites showing other evidences of the action of the drug. The chromatin may be situated in one or more of the fragments of protoplasm or lie free within the erythrocyte, generally near the periphery. The extrusion of the chromatin from the plasmodium is quite frequently observed at this stage of development. The vesicular portion of the nucleus of the plasmodium is not visible. It is evident from the morphological appearances described that these fragmented plasmodia have been destroyed by the drug but others are observed which present no evidences of fragmentation but which are undoubtedly injured, though to a less degree, by the drug. These plasmodia are irregular in shape; the protoplasm takes a deeper blue stain than normally; while the chromatin is collected in one or two large clumps near the periphery of the organism. Evidence of normal division of the chromatin is almost lacking, and in some instances, extrusion of this substance has occurred, thus proving that quinine possesses the power of rendering the plasmodia sterile. The pigment is markedly decreased in amount and generally arranged in irregular clumps near the periphery of the plasmodium. The organisms, which show little evidence of ameboid activity, are smaller than normal and the deeper blue staining of the protoplasm is probably due to a concentration of the latter. In the unfragmented plasmodia there is no evidence of the vesicular portion of the nucleus.

Extracellular plasmodia undergoing fragmentation, or isolated fragments of such plasmodia, are frequently observed at this time in stained preparations. The fragments stain a deep blue and may be devoid of pigment or filled with it, while rarely a clump of chromatin may be observed within a fragment. The occurrence of these extracellular parasites brings up the question of their origin. Are they extruded from the infected red corpuscle or are they liberated by the disintegration of the latter? The evidence pointing to their extrusion is slight and inconclusive and I believe that they are liberated by the disintegration of the infected erythrocyte. That quinine produces degeneration of the infected red corpuscle is evidenced by the occurrence of polychromatophilia and basophilia after its administration to a much greater extent than before.

If quinine has only been administered for a day or two the preparations will show plasmodia normal in appearance, but they are few in number in comparison with

the injured plasmodia. The plasmodia which are able to resist the action of quinine until fully developed present strong evidence of the inhibitory action of the drug upon normal schizogony. Even at this late stage some of the plasmodia are found to be undergoing fragmentation but the majority are evidently in the presegmenting stage. In the latter the protoplasm stains a distinct blue, the organism appears shrunken, and the pigment is small in amount and collected in large granules or clumps about the periphery of the parasite. The chromatin has increased but little in amount, stains a dark violet, and is arranged in two or three irregular masses within the protoplasm. The small amount of chromatin and its arrangement are eloquent of the action of quinine in preventing typical sporulation, for in the normal plasmodia at this stage of development the chromatin has increased enormously in amount, stains a brilliant crimson, and is distributed in numerous small clumps throughout the protoplasm.

Extracellular fragmented plasmodia are still present but are not nearly so numerous as in earlier stages of development of the parasites. Indeed, fragmentation due to quinine is most frequent in the early pigmented stages of development and this may be said to be the characteristic form of degeneration when the parasites are from one-half to three-quarters developed.

In those plasmodia which are undoubtedly preparing to segment, the protoplasm stains a diffuse blue, the lines of cleavage of the developing merozoites being indistinct or irregular. The chromatin is very small in amount as compared with normal plasmodia, and is collected in the protoplasm in very irregular masses varying much in size and situated at irregular intervals within the protoplasm. Some plasmodia show only one or two such masses of chromatin while others may contain several, but they are always atypical in appearance and stain a very dark violet instead of the brilliant crimson color observed in normal plasmodia. The pigment is small in amount and generally arranged about the periphery of the plasmodium.

The sporulating plasmodia, which have partially resisted the action of quinine during their development, present morphological changes of great interest. The number of merozoites is generally reduced, some of the plasmodia showing only six or eight; the contour of many of them is irregular, and many appear devoid of chromatin, consisting of protoplasm only. The merozoites which contain chromatin are often irregular in shape, and in most of them there is no trace of the unstained portion of the nucleus, while the chromatin is atypical in appearance, staining an almost black color, and consisting of two or more granules instead of the single large dot so characteristic of the normal merozoite. Parasites are not infrequently observed undergoing sporulation in which only two or three of the merozoites contain chromatin, the remainder being simply masses of blue-stained protoplasm, irregular in shape, and sometimes much vacuolated. In some of the plasmodia at this stage of development a process of partial sporulation appears to have occurred, a portion of the organism consisting of well-defined merozoites, while the remainder consists of a mass of protoplasm in which no trace of division into merozoites can be demonstrated. Such organisms forcibly suggest the bodies described by Schaudinn as macrogametes undergoing parthenogenesis. I have never observed such parasites in the blood of patients who have not taken quinine.

The merozoites which are devoid of chromatin are generally irregular in shape and stain a deep blue or bluish-green. A few granules of residual pigment may sometimes be observed within them, indicating a fragmentation of the plasmodium before sporulation is completed. In fact, I believe that most of the merozoites devoid of

chromatin originate from plasmodia which were in an almost perishing condition when sporulation occurred. If this be the true explanation of the phenomenon, it is evident that quinine possesses the power of injuring every developmental stage in the human life-cycle of Plasmodium vivax.

Normal sporulating plasmodia may be observed in small number in the blood of patients who have not been treated with quinine for more than a day or two, along with those presenting the morphological changes described.

The morphological changes produced in the young gametes are similar to those produced in the schizonts, and, for this reason, do not need to be further described. There are the same changes in the staining reaction of the protoplasm and chromatin; the same lack of evidence of increase in the amount of the latter; and the same absence of the vesicular portion of the nucleus. The gametes are apparently not affected by the drug after they have developed much pigment and in preparations from patients who have suffered for some time from the infection, gametes normal in appearance may be observed along with numerous full-grown or sporulating plasmodia presenting the most profound evidence of the action of the drug.

The morphological changes produced in Plasmodium vivax by quinine, as shown in stained specimens, may be summarized as follows:

1. Changes in the staining reaction of the protoplasm and chromatin.

2. Fragmentation of both the protoplasm and chromatin.

3. Prevention of normal increase in the amount of chromatin and of normal division.

4. The production of atypical sporulating plasmodia, in which many of the merozoites appear devoid of chromatin.

5. Similar changes in the young gametes as regards the protoplasm and chromatin.

Plasmodium malariae.—The changes produced in Plasmodium malariae by quinine are very similar to those already described as occurring in Plasmodium vivax as regards morphology and I cannot confirm the observations of Antolisei that the older quartan plasmodia are not affected by this drug. While there is some slight evidence that this species is more resistant to the action of quinine than is Plasmodium vivax, it is nevertheless true that the drug acts upon every stage of schizogony.

Fresh preparations.—In the young "ring-forms" marked increase in ameboid motion is noticed after the administration of the drug, succeeded, as in Plasmodium vivax, by decreased motility, and finally, by cessation of all motion. In the young pigmented forms fragmentation is often observed and ameboid activity is absent. The pigment is small in amount and immotile. The half and three-quarters grown

organisms present a very granular protoplasm, fragmentation is common, and the pigment is reduced in amount. In the presegmenting parasites the reduction in the amount of pigment is very noticeable, while the distribution of this substance in the protoplasm is irregular and atypical. The sporulating parasites show a reduced number of merozoites and the latter appear granular and very refractive.

Stained preparations.—Practically the same changes are observed as occur in Plasmodium vivax. The protoplasm stains a deep blue and the chromatin takes a dark violet color. The latter often appears fragmented and the amount present is much smaller than normal in all stages of development of the parasite. Fragmentation of the protoplasm and extrusion of the chromatin are frequently noticed and even in the adult parasite fragmentation is far from being rare. The vesicular portion of the nucleus is absent from the pigmented organisms and the amount of pigment is reduced. In the presegmenting parasites the chromatin is small in amount and arranged in two to three irregular clumps near the periphery of the plasmodium. Extracellular fragmenting plasmodia are frequently observed and appear as do those of Plasmodium vivax.

The sporulating quartan plasmodium stains diffusely blue after administration of quinine; the number of merozoites is reduced; while in many organisms there occurs an irregular mass of protoplasm which shows no trace of division and is devoid of chromatin, along with a few atypical merozoites. The merozoites are irregular in contour and are often devoid of chromatin, while the protoplasm stains a deep blue color.

As in tertian infections, apparently normal plasmodia are observed along with the degenerative forms, provided the quinine has not been administered for more than three or four days.

The effect of quinine upon the gametes of Plasmodium malariae is similar in every respect to its effect upon the gametes of Plasmodium vivax, the young gametes only being acted upon by the drug, so far as can be ascertained.

Plasmodium falciparum and Plasmodium falciparum quotidianum.—The effect of quinine upon the estivo-autumnal plasmodia is the same in both species, for which reason they will be considered together. I have studied the action of the drug upon every stage in the human life-cycle of these plasmodia with the exception of the fully developed pigmented organisms and the sporulating stage. As is well known, the latter forms are very seldom observed in the peripheral blood, it being necessary to puncture the spleen in order to obtain material for study, and because of this my observations have been so limited as regards these stages in the development of the estivo-autumnal plasmodia that I do not feel justified in drawing any definite conclusions from them. In one fatal case of pernicious tertian estivo-autumnal malaria to which quinine had been administered some hours before death, the splenic smears showed many sporulating plasmodia, some of which presented a smaller number

of merozoites than normal, while the morphological changes were similar to those described as occurring in Plasmodium vivax and Plasmodium malariae.

Fresh preparations.—In preparations of fresh blood the estivo-autumnal plasmodia while in the "ring-stage" appear more refractive after the administration of quinine than normally, while the ameboid activity is greatly stimulated. The "ring-form" is often lost, the parasites becoming discoid in shape, as described by Marchiafava and Bignami, or the contour becomes irregular so that they resemble the young hyaline stage of the benign tertian plasmodium. I have never observed the extrusion of the plasmodia from the erythrocyte which has been described by Marchiafava and Bignami as characteristic of the action of quinine upon these species of plasmodia. The increased ameboid activity of the plasmodia causes them frequently to change their position in the infected corpuscle, and they sometimes move very rapidly from one portion of the red cell to another. After a variable time the movements become slower and finally cease.

The protoplasm of the plasmodia appears more granular than normal, while the outline of the parasite, when motion has ceased, is very sharply defined. Upon the pigmented "rings" and the larger pigmented bodies observed in the peripheral blood, quinine appears to produce a granular degeneration of the protoplasm and a great increase in the refractive index of the plasmodia. Ameboid motion is absent and the outline of the organism is very sharply cut. The amount of pigment is apparently normal.

Stained preparations.—That quinine produces marked morphological changes in the estivo-autumnal plasmodia is amply shown by the study of stained preparations of blood from patients to whom this drug has been administered. Marchiafava and Bignami concluded from their studies that no marked morphological changes occurred in these plasmodia after the administration of quinine, while pigment formation ceased after such administration. I cannot confirm their conclusions, for I have found that quinine produces changes in the morphology of these plasmodia similar to those occurring in Plasmodium vivax and Plasmodium malariae and that pigmented plasmodia are often observed in the blood for several days after the administration of the drug.

The protoplasm of the "ring-forms" stains more intensely blue than normally while the chromatin stains a very dark violet color. The unstained area surrounding the chromatin is generally absent and the young plasmodium often consists of a stained mass of protoplasm, oval or round in shape, containing a small clump of chromatin. Many of the plasmodia in this early stage of development are irregular in form, due to the stimulation in ameboid activity. Extrusion of the chromatin from the plasmodia is rarely observed.

In the larger pigmented plasmodia the morphological changes produced by quinine, as shown in stained preparations, are similar in every respect to those observed in Plasmodium vivax and Plasmodium malariae, except that fragmentation is less common and extracellular forms are generally absent. I do not feel justified in describing the changes produced in the sporulating organisms, as my material has been so limited since I began the study of this subject. In the one case I have already mentioned that the morphological changes observed were identical with those occurring in the other species of human plasmodia, altho more normal plasmodia were observed.

I have not been able to observe that the administration of quinine produces any

morphological changes in the fully developed estivo-autumnal gametes (crescents) but the drug affects the early stages of development of this type of the plasmodia in the same manner as it does the young gametes of the other species of plasmodia.

The summary given of the changes produced by quinine in Plasmodium vivax serves equally well for Plasmodium falciparum and Plasmodium falciparum quotidianum, so far as I have been able to determine, with the exception that in the estivo-autumnal plasmodia the drug less frequently causes fragmentation.

Practical deductions.—It is evident from the observations noted that quinine, whenever given, is effective in destroying the plasmodia, but it is also evident that the drug is most effective if given in divided doses at regular intervals, thus keeping the blood charged with it. Given in this manner the plasmodia are continually exposed to the action of the drug and the morphological changes observed in the plasmodia prove that the drug is capable of destroying them at every stage in their life-cycle in man, with the possible exception of the sporulating stage, but even in this stage it produced an atypical division of the parasite, resulting in a lessened number of merozoites and sterility of many of the latter.

If the drug be administered in one large dose just before sporulation very many of the merozoites are at once destroyed but those which are able to survive for a few hours develop and sporulate in a normal manner, the drug having been excreted before the cycle of development has been completed, some stages escaping entirely from the effect of the drug. If, on the other hand, after a moderate-sized initial dose, the drug be continued at intervals of three or four hours, those parasites which have escaped the first dose are not free to develop normally but are continually exposed to the action of the drug, which is always present in the blood.

My observations show that the plasmodia under such conditions are injured during every stage of their growth, many perishing before sporulation, while those that sporulate do so in an atypical manner. The conclusions drawn regarding the time of the administration of quinine, as the result of the study of the morphological changes produced by the drug, are not only justified theoretically, but have the confirmation of actual clinical experience.

I have the clinical records of over 2,000 patients suffering from the various types of malarial fever, in which quinine was given in divided doses at regular intervals. In all of these cases recovery from the infection was prompt and more rapid than in similar cases in which the drug was administered in one large dose prior to, or immediately following, sporulation. In the tertian infections a second chill very rarely followed the administration of the drug, altho most of the cases showed a rise of temperature upon the day of the expected paroxysm. In only about 20 per cent of these, however, did the temperature reach 101° F., and in most of them it did not rise above 100° F. In the quartan infections, a second paroxysm never occurred if the drug was administered in this way, provided the initial dose was given within 12 hours after the first paroxysm. In the estivo-autumnal infections the method of administering quinine in divided doses, at regular intervals, is the only one which should be employed, for it is generally impossible to ascertain the exact time of the expected paroxysm in most of these infections, so that it is impossible to give the drug in one large dose, in the expectation of destroying the merozoites before they infect the erythrocytes. In the many hundred cases of estivo-autumnal malaria in which I have seen quinine administered in divided doses, the results have been most satisfactory, provided the treatment was begun before the appearance of pernicious symptoms, and even in the pernicious cases, the best results are secured by giving the drug hypodermically or intravenously, in divided doses. In ordinary cases of estivo-autumnal malaria the administration of the drug in divided doses results in a fall of the temperature to normal within three days, and in the milder infections the fever generally disappears within two days. In estivo-autumnal infections quinine should never be administered in one large dose during the 24 hours, as such treatment invariably results in the febrile condition persisting for several days, during which time pernicious symptoms may develop. I have observed very serious results follow the administration of quinine in this manner, some of the patients so treated dying from pernicious attacks which might have been controlled had the drug been administered in divided doses at regular intervals.

PART II.

STUDIES UPON INTRACORPUSCULAR CONJUGATION IN THE MALARIAL PLASMODIA.

In previous communications[1] I have described a process of conjugation observed in the malarial plasmodia, occurring within the infected erythrocytes, and apparently of importance in the life-history of the parasites, as it occurs very frequently and is uniform in its phenomena.

This process was first described, so far as I have been able to ascertain, by Mannaberg,[2] who considered that it was the first stage in the development of the crescent form of the estivo-autumnal plasmodia; while later, the same phenomena were described by Ew ng,[3] but were considered by him as of rare occurrence and of little importance in the life-cycle of the plasmodia.

Mannaberg described the process very accurately. He says, regarding the method of reproduction of the malarial plasmodia: "The reproduction of the malarial parasites takes place through a simple segmentation (spore formation) in the developed condition, probably also by the formation of spores after previous conjugation and encystment." He describes the occurrence within the erythrocytes of two or more plasmodia and distinguishes multiple infection of the red corpuscles from the true conjugation forms. Regarding the latter he says: "These paired parasites occupy usually the edge, less frequently the center of the blood corpuscle. In some pairs the protoplasmic membranes are clearly seen lying close to one another; at other times they are hazy or hardly to be recognized, so that in the latter case one gets the impression that through coalescence the two parasites have produced a new form, which in its structure betrays its origin." Again he says: "Returning now to the paired ameboid malarial parasites, the analogy of the zoölogical facts just mentioned must lead us to the opinion that we have possibly here to do with a process of conjugation." Mannaberg gave this subject much attention and was able to observe actual blending of the two plasmodia with the production of a new and larger organism. Regarding this observation he states: "I gave, during this last summer, very careful attention to fresh preparations, especially to the double invasion of the red blood corpuscles, viewing them for long periods, in order to see whether one could observe the process of coalescence of two parasites. My efforts were successful, for I was able repeatedly to see how two parasites coalesced and formed a larger body. After this there can be no doubt that the ameboid parasites, coalescing with each other, can from larger bodies."

The observations of Mannaberg are of great interest and value, for while he erred in interpreting conjugation as preliminary to the formation of the crescents, or gametes,

[1] Craig, *Amer. Med.*, 1905, 10, pp. 982, 1029; *Jour. Infect. Dis.*, 1907, 4, p. 10; *Inter. Clinics*, 1907, 3, p. 273; *Philippine Jour. Sci.*, 1906, 1, p. 523.

[2] "The Malarial Parasites," *New Sydenhem Soc.*, 1894, p. 241.

[3] *Clinical Pathology of the Blood*, New York, 1903, p. 454.

he gives the first accurate description of the process and observed it in the living organisms, as well as in stained preparations.

Mannaberg's observations were made upon estivo-autumnal plasmodia, while those of Ewing were made upon both the tertian and estivo-autumnal plasmodia. The latter observer described the process as occurring but rarely in these infections and speaks of conjugation between both the young plasmodia and larger, pigmented forms. His description of the process, in so far as it concerns the young, unpigmented plasmodia, is excellent, but it is evident that he confused multiple infection of the erythrocyte with conjugation, for true conjugation occurs in the hyaline plasmodia only before the formation of pigment, and the large pigmented tertian conjugation forms described by him were undoubtedly instances of the multiple infection of the red cell by two tertian plasmodia which had developed pigment. Ewing was undecided regarding the significance of the process in the malarial plasmodia, regarding which he says: "The extent and significance of this form of conjugation it is difficult to determine. In the cases in which it can be profitably studied, parasites are very abundant, and in most cases few indications of the process can be detected. It is, therefore, probably not essential to sporulation, and when parasites are scanty the chances of finding typical examples of conjugation pairs are greatly reduced, but the peripheral blood may not be a complete index of the process in the internal viscera. It seems probable that conjugation occurs in the first generation of the infection, and becomes less frequent as the disease progresses, the infection in the human host thereby tending to limit itself."

It is interesting to observe that Ewing believed that the process has something to do with the limitation of the infection, altho he evidently did not regard it as a means by which the infection is kept alive within man.

For over seven years, in the course of other studies upon the malarial plasmodia, I have observed the occurrence of this form of conjugation in the plasmodia of man and have carefully studied its various stages. The process occurs in every malarial infection in which quinine has not been given early; it occurs in recurrent attacks in which acute symptoms have been present for several days; and in it I believe that we have the most rational explanation of latency and recurrence in malarial disease. The material for my studies upon this subject has embraced over 500 cases of malarial fever observed in this country in soldiers returning from the Philippine Islands, and nearly 200 cases observed in the Philippines. Of these, 175 were latent infections (infections without symptoms), about 200 were acute initial infections, and the remainder recurrent infections, one or more paroxysms having occurred days or weeks before the patient came under my observation.

Conjugation and the species of plasmodium.—Intracorpuscular conjugation occurs in all of the species of malarial plasmodia infecting man but is most frequently observed, and most easily studied, in

infections with the estivo-autumnal plasmodia, the reason being the greater resistance of these species to treatment and, therefore, the longer duration of the infection. There is no reason to believe, however, that the process is less frequent in infections with Plasmodium vivax and Plasmodium malariae, which have had no treatment, altho it is less frequently observed. This form of conjugation is a normal phase of the life-cycle of all species of human malarial plasmodia under certain conditions, but, as will be seen later, it does not occur in infections of slight duration, or in infections in which proper treatment has been initiated early in the course of the disease.

Time of occurrence.—Intracorpuscular conjugation never occurs during the first few days of an acute malarial infection and for this reason it is not observed in initial acute malarial attacks, or in recurrent cases in which quinine has been administered promptly. I have never observed the process in cases in which but two or three paroxysms had occurred prior to treatment, but after the infection has persisted for a week or more, intracorpuscular conjugation is invariably observed. An exception to the statement as regards quinine is found, of course, when the dose of that drug is insufficient, and it is because of the resistance of the estivo-autumnal plasmodia to quinine that we so frequently observe this form of conjugation even in cases which have been treated for short periods of time. In all acute and recurrent malarial infections, which have persisted for a week or more, instances of intracorpuscular conjugation will be found, if carefully searched for, in the peripheral blood. In latent infections the process is rarely observed in the peripheral blood, and only in long-continued infections.

The process of intracorpuscular conjugation is most frequently observed in infections which have lasted for a long time, and in which there have been several recurrences. Thus in estivo-autumnal infections, especially those presenting pernicious symptoms, the process is almost always observed, as these infections are generally of long duration and are sure to recur unless very thoroughly treated with quinine. In all infections in which sporulation has occurred several times, and where the plasmodia are very numerous, intracorpuscular conjugation is observed. In those pernicious cases in which the symptoms develop suddenly during a short initial attack

of the fever, this form of conjugation is never observed, so far as I have been able to determine. As regards the time of occurrence of intracorpuscular conjugation we may state that it occurs only after the plasmodia have multiplied in the usual manner for several generations, and thus it is observed only in those malarial infections which have persisted for some time. The process is evidently intended to preserve the race of plasmodia from extinction as the result of repeated multiplication. In one case of fatal pernicious malaria due to the tertian estivo-autumnal plasmodium, in which the parasites had multiplied to such an extent that almost every other red corpuscle was infected, examples of intracorpuscular conjugation were observed in practically every microscopic field.

Multiple infection of the erythrocyte and intracorpuscular conjugation.—Multiple infection of the erythrocyte by the malarial plasmodia is of very frequent occurrence, especially in infections with Plasmodium falciparum quotidianum, and should be carefully distinguished from intracorpuscular conjugation. Cropper[1] describes multiple infection of the erythrocyte in a case of pernicious malaria and states that when such multiple infections occur the plasmodia develop as usual and go on to sporulation. Cropper thinks that this observation precludes the occurrence of the form of conjugation described in this paper, but if he had read Mannaberg's paper, and my original description of this form of conjugation, it would have been apparent to him that we had not confused conjugation with multiple infection, and that it would be impossible to mistake the two phenomena. In my original description of intracorpuscular conjugation I say:[2] "As is well known, it is very common in specimens of blood from patients suffering from estivo-autumnal infections to see cells containing two or more "ring-forms" of the plasmodia: such parasites, however, are not always conjugating forms." Again, in the same contribution, I say: "We must admit that in some instances two parasites develop independently, so far as we can determine, for I have seen red corpuscles containing two plasmodia at different stages of development, and this is not uncommonly observed in tertian infections."

From these quotations it is evident that I did not confuse multiple infection of the erythrocyte with intracorpuscular conjugation, as

[1] *Proc. Soc. Trop. Med.*, 1907–1908, 1, p. 145. [2] Craig, *Amer. Med.*, 1905, 10, pp. 982, 1029.

Cropper appears to believe. Multiple infection bears no resemblance whatever to intracorpuscular conjugation, as will be appreciated when we consider the morphological phenomena of the latter, and it would be impossible for anyone who has studied the malarial plasmodia concerned in this process to confuse it with such infection.

FIG. 1.—Blood smear from case of estivo-autumnal malaria, showing multiple infection of the red cell with two "ring-forms" and a sporulating plasmodium. Note the small size of the "rings" and the minute dot of chromatin.*

In multiple infection, while the parasites may lie in apposition, there is no evidence of the union of the protoplasm and nucleus, and, as has been known for years, the two organisms, if they survive, develop in a normal manner and eventually sporulate. In intracorpuscular conjugation there is a visible union of the two plasmodia and the production of a single and larger organism. Mannaberg was the first to demonstrate this fact in living plasmodia, and I have been able to confirm it repeatedly. In addition every stage of the process is visible in stained specimens and it is impossible to confuse these stages with multiple infection of the red corpuscle, if one has had experience in the examination of malarial blood. The photomicrographs which accompany this study show that in intracorpuscular conjugation we are not dealing with multiple infection of the erythrocyte.

Morphology of intracorpuscular conjugation.—This form of conjugation occurs only between two young hyaline forms of the plasmodia, indistinguishable in size and structure. The process, therefore, is isogamous in nature, the conjugants being undifferentiated. As the phenomena of this form of conjugation are similar in all of the species of human plasmodia, I shall describe it as observed

* NOTE.—I desire to express my thanks to Dr. William M. Gray, of the Army Medical Museum, for the photomicrographs which accompany this contribution. The magnification in all the photomicrographs, with the exception of Fig. 12, is ×1500. Figure 12 is ×1200. Photomicrographs 10, 11, and 12 are inserted for purposes of comparison with the pigmented conjugation form illustrated in Fig. 9.

in infections with Plasmodium falciparum, as it is very frequently observed in such infections.

The process invariably occurs between two unpigmented "ring-forms" and is first evidenced by the union of the protoplasm of the two conjugants. Red corpuscles are observed containing two "ring-forms" lying in apposition and careful examination will demonstrate that at the point of contact there is undoubted union of the protoplasm of the two organisms. In fresh preparations the process may be followed in rare instances, and I have several times been so fortunate as to witness the complete union of the conjugants. In the fresh blood conjugation commences by the gradual approach of the two organisms until they are finally in apposition, the ameboid processes of the adjoining segments of the "rings" merging rapidly, so that in less than a moment there is no distinction between the two parasites at the point of apposition. A gradual blending of the protoplasm now occurs, until in a period varying from ten minutes to half an hour, all trace of two distinct organisms has been lost, and only one slightly larger plasmodium can be seen within the erythrocyte. In such preparations, of course, no structural details can be made out, but it is certain that while the two organisms concerned in conjugation become one in the time mentioned, the process is not completed, for, as shown in stained preparations, changes occur in the nucleus which require for their completion several hours. It is probable that intracorpuscular conjugation is not completed much within 24 hours.

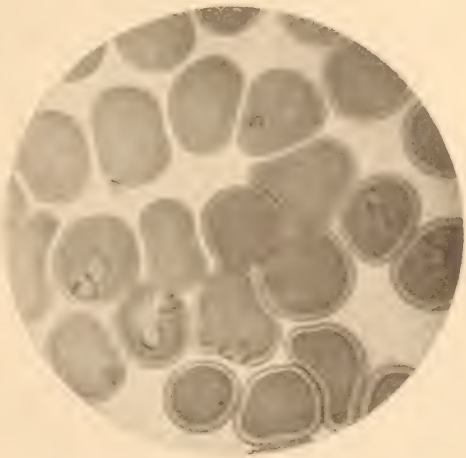


Fig. 2.—Estivo-autumnal infection. This specimen shows multiple infection of the erythrocyte and intracorpuscular conjugation, in which the conjugants are in the first and second stages of the process. Note the "rings" with the double dot of chromatin, and the two parasites which are in the first stage, before the complete union of the rings.

In stained preparations it is usually observed that the primary

union of the conjugating organisms occurs at some point remote from the nuclear chromatin. The latter may be situated at any portion of the plasmodia but in most instances the chromatin mass is situated on the outer periphery of the two "ring-forms" opposite the point of apposition. With the Wright stain the protoplasm of the plasmodia stains the usual blue color, the chromatin mass a brilliant crimson, while the vesicular portion of the nucleus remains unstained. Not infrequently "ring-forms" are observed in which the chromatin masses are apparently united but I do not consider such forms as conjugating organisms. They are independent plasmodia which are accidentally in contact in this manner. Careful examination will demonstrate that there is no union of the chromatin or of the protoplasm. That the apposition of the conjugants results in a true union of protoplasm is proven by the fact that there is no evidence of the outline of the "rings" at this point, or of overlapping. The chromatin masses are distinct and alike in appearance and the outline of the "rings" is preserved except at the point of contact (see Figs. E, 3, and 5).

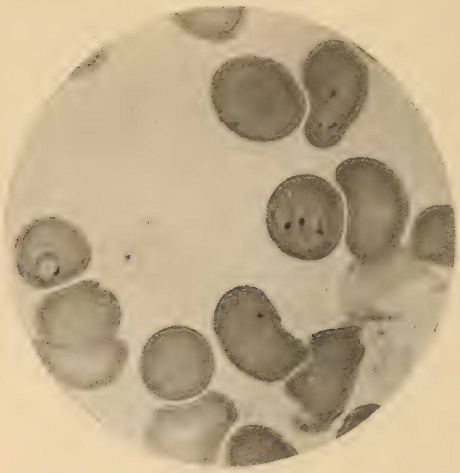


FIG. 3.—Estivo-autumnal infection. Showing multiple infection of the erythrocyte and one conjugation form which is distinguished by its large size and two masses of chromatin. In this organism protoplasmic union is complete and the resulting "ring" is twice as large as those in the doubly infected cell.

The initial stage of conjugation just described is followed by complete protoplasmic union, the two chromatin masses, in stained preparations, being observed within a single organism. While various examples of this stage of conjugation are observed in stained specimens, in all of them the slightly larger organisms resulting from the union of the two "rings" preserve their "ring" shape, the distinctive features being the increase in size and the presence within the organism of two distinct chromatin masses of equal size, and sur-

rounded by an unstained area due to achromatic substance. The chromatin masses vary considerably in situation, sometimes being opposite one another, near the periphery of the organism, or lying almost in contact. All of the stages of union of the protoplasm as observed in stained specimens tend to show that the union is accompanied by a gradual absorption of the central portion of the adjoining segments of the "rings" until only a fine thread of protoplasm remains which eventually is absorbed and we have the production of the single, but larger "ring-shaped" plasmodium (see Fig. F).

The process of intracorpuscular conjugation is completed by the apparent union of the nuclei of the two plasmodia, which is evidenced in stained specimens by the union of the chromatin masses, accompanied by apparent reduction phenomena, and the extrusion of minute granules of chromatin. In such preparations plasmodia are observed illustrating various phases in the union of the chromatin masses and there can be no doubt that this union is marked by changes in the appearance of the chromatin and in its amount, while very frequently small dots of this substance are observed lying outside of the organism but within the protoplasm of the erythrocyte (see Fig. 7).

In many specimens it will be observed that the chromatin in one of the masses, instead of being collected in a compact mass, as it is in the other, is separated into very fine filaments or granules, and distributed over a segment of the "ring," thus bringing it nearer to the other mass of chromatin. This is apparently the first stage of nuclear union, and is observed only when the two chromatin masses have been situated opposite one another or some distance apart. Sometimes, under such circumstances, both masses of chromatin are broken up into filaments or granules (see Figs. I, 6).

In those instances in which the chromatin masses have been situated near one another, union appears to be effected by a lengthening of the masses until they meet, while in the first instance mentioned, the thinned mass of chromatin apparently travels around the intervening segments of the "ring" until it comes in contact with the other mass and unites with it (see Figs. J and K).

After the union of the two masses of chromatin, which generally

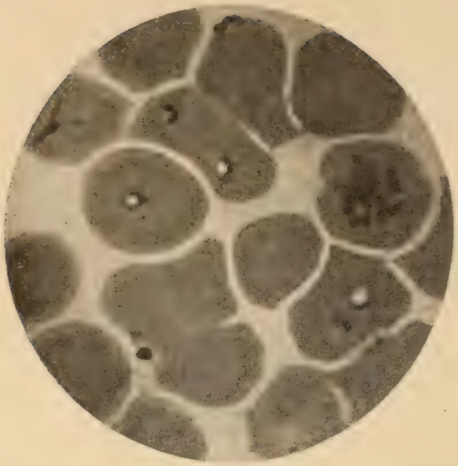


FIG. 4.—Estivo-autumnal infection. Showing various forms of conjugating organisms and a sporulating parasite. It will be seen that the chromatin dots in some of these organisms are almost united, while in others they have united, giving rise to a peculiar cupped appearance of this portion of the nucleus.

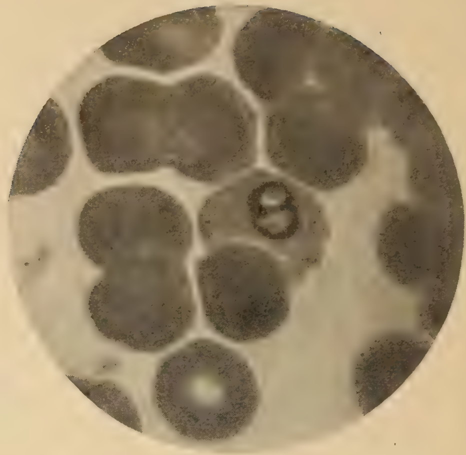


FIG. 5.—Tertian infection. Conjugating parasites in which protoplasmic union is almost complete, and the portions in apposition are beginning to be absorbed. The two chromatin masses are somewhat indistinct but are still separated.

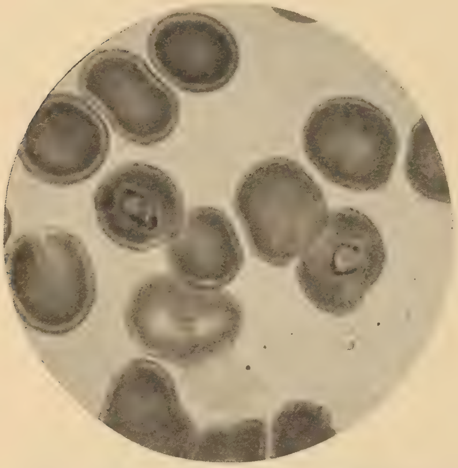


FIG. 6.—Tertian infection. Two conjugating forms in which the process is almost completed. Note the character of the chromatin masses.

FIG. 7.—Tertian infection. A conjugating form in which the two chromatin masses are distinct and the portions of protoplasm in apposition have become absorbed, resulting in the formation of a large ring-like body with two chromatin masses. A small mass of extruded chromatin is seen lying in the infected cell.

occurs at some portion of the periphery of the organism, the chromatin takes up a position within the "ring," generally at the center, and is surrounded by an achromatic zone. When conjugation is complete the appearance of the chromatin is that of a compact, circular mass, staining a deep red, and of considerable size. The entire organism resembles very markedly the first stage in the growth of the malarial gametes, save that the organism is much larger and the chromatin mass is more deeply stained and of twice the size of the chromatin mass in the gametes.

Fig. 8.—A large pigmented form resulting from conjugation. Tertian infection. This body is extracellular, has five large clumps of chromatin, and a very small amount of pigment. It is typical of the large pigmented forms which result from conjugation, and entirely distinct from the presegmenting form of the tertian plasmodium which is illustrated in Fig. 12.

In all stained specimens of blood from cases in which the malarial plasmodia have undergone schizogony for several generations, the stages of conjugation described can be observed, and it is evident that the process consists in a union of the protoplasm of the conjugants, followed by the union of the nuclei, accompanied by the extrusion of a small portion of the nuclear chromatin.

It is not necessary to describe in full this process as it occurs in tertian and quartan infections, as it does not differ in any particular from the same process in the estivo-autumnal infections. Conjugation is completed during the hyaline stage before the formation of pigment, in both types of infection, but it is not always confined to the "ring-forms," for not infrequently conjugating forms are observed in which the active ameboid motions of the parasites have distorted the "rings," causing irregularly shaped organisms.

After he completion of conjugation the single parasite resulting develops within the erythrocyte and finally destroys it, becoming free in the blood. These developing parasites are distinguished from

other forms of the plasmodia by the smaller amount of pigment, and, in stained specimens, by the collection of the chromatin in two or more irregular, deeply stained masses, distributed about the center of the organism (Figs. 8, N, O, and P).

In some instances the chromatin masses are numerous and are collected at one side of the organism, giving rise to an appearance suggesting sporulation. Such forms are very suggestive of the parthenogenetic gametes as pictured by Schaudinn in the contribution I have already referred to (see Fig. 9). The large pigmented forms are observed lying free in the blood plasma very frequently when conjugation is present but they never sporulate, nor have I ever observed exflagellation in any of these bodies. To my mind such forms as are illustrated in Fig. 9 are preparing for sporulation and are probably the forms which give rise to the young parasites which are responsible for recurrences, but they are certainly not macrogametes undergoing parthenogenesis, as they occur only *when conjugation is present*, and very frequently *before* gametes have appeared in the blood. In the estivo-autumnal infections these large pigmented forms are only observed in the blood obtained by splenic puncture, and they are not observed in any form of malarial infection in which intracorpuscular conjugation is absent. The growth of the parasite resulting from conjugation is rapid while it is contained within the erythrocyte but after being liberated there are no evidences of further growth and it is probable that the parasite then enters upon a resting stage, which may last for days or weeks, according to the conditions present.

It is these pigmented bodies which I believe were considered by Schaudinn to be macrogametes undergoing parthenogenesis, as his description of the presegmenting form of the macrogamete answers as well for the pigmented stages of the conjugating plasmodia. That this form of conjugation is of significance in the life-cycle of the plasmodia is evident from the occurrence of the process only in malarial infections which have existed for some time; the very uniform phenomena observed; the occurrence of developing forms dissimilar from other forms of the malarial plasmodia in the blood of patients showing conjugation; and the resemblance of the process to conjugation in other of the protozoa. The fact that typical conjugating

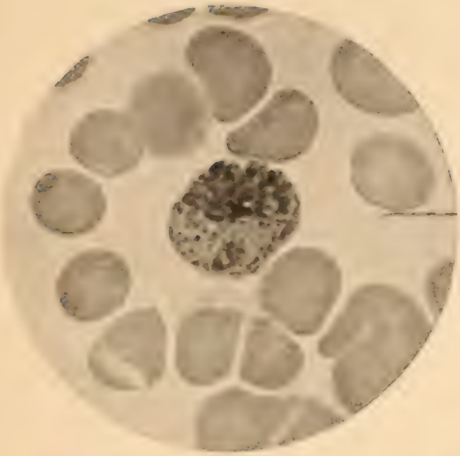


Fig. 9.—Tertian infection. Large pigmented form, the result of conjugation. This is the form which may have been considered by Schaudinn as a macrogamete undergoing parthenogenesis. Note the appearance resembling sporulation, the chromatin being divided into small masses and collected upon one side of the organism. These forms are very rare in the peripheral blood.

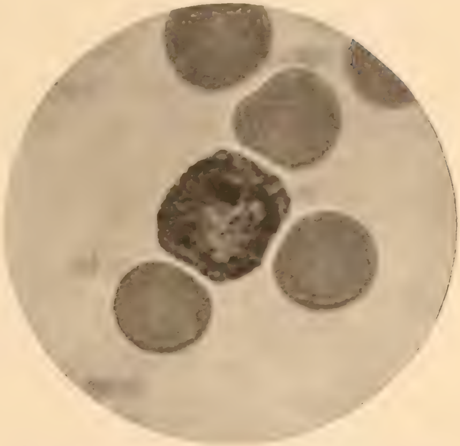


Fig. 10.—Tertian infection. A tertian macrogamete. Note large size, dense mass of chromatin, and large number of coarse granules of pigment.

Fig. 11.—A tertian microgametocyte. Note the character of the chromatin mass as compared with that of the macrogamete; the finely granular pigment, which is also smaller in amount, and the poor staining quality of the parasite.

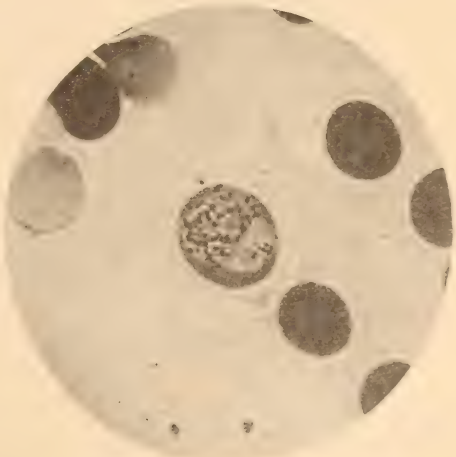


Fig. 12.—A typical presegmenting tertian plasmodium. Note the numerous small masses of chromatin and their distribution, as compared with the conjugation form shown in Fig. 8.

plasmodia are observed in the blood when all other hyaline forms have been replaced by pigmented forms, proves that the process has nothing to do with multiple infection of the erythrocyte, and that it requires several hours for its completion. For instance, in a typical single tertian infection, while all of the plasmodia undergoing schizogony have reached the presegmenting or three-quarter-grown stage, the hyaline conjugating bodies may also be present, and these are the only hyaline plasmodia observed. The presence of these forms in cases in which nearly all of the plasmodia have become pigmented often leads to the diagnosis of a double infection. So far as I have been able to observe neither the conjugating plasmodia, nor the large pigmented parasites developing from them, are affected by quinine. At least the changes which I have described as occurring in the schizogonic forms in Part I of this contribution I have never observed in the conjugating organisms.

Significance of the process of intracorpuscular conjugation.—I shall not, in this contribution, discuss the significance of conjugation in the Protozoa in general, which I have considered elsewhere,[1] but will simply state that the process is of great importance in the life-history of the organisms in which it has been observed. While the process of intracorpuscular conjugation in the malarial plasmodia has no exact analogue in other closely related parasites, it is but reasonable to believe that it is of importance in the life-history of the species of plasmodia in which it occurs. The morphological changes observed during the process of intracorpuscular conjugation are similar to those observed during the conjugation of other protozoan organisms and are of such a nature that they cannot be explained on the theory that they are plastogamic in nature, or accidental in occurrence. While plastogamy is very common in the Protozoa, it is not followed, as in intracorpuscular conjugation, by the development of forms of the parasite different in morphology from those usually observed—forms which are observed only after this process has occurred. The conjugating plasmodia do not separate after a period of time, but, as I have observed in fresh preparations, gradually blend and become one organism, which finally develops into the large pigmented body described. It is evident that the malarial plasmodia

[1] *Jour. Infect. Dis.*, 1907, 4, p. 108; *The Malarial Fevers, Haemoglobinuric Fever, and the Blood Protozoa of Man*, New York, 1909.

must exist in some form within man during the latent period of infection and between recurrences. I have previously shown that in latent infections (infections without symptoms) the plasmodia undergo normal schizogony within the spleen, but, while this is so, it is impossible to believe that this process can go on for weeks, and even months, without multitudes of plasmodia being produced, and, consequently, symptoms of the disease.

Various theories have been adduced to explain the occurrence of relapses in malaria, after long periods of time, and the phenomenon of latent malarial infection. Bignami considered that the plasmodia existed somewhere in the body in a latent form, perhaps encapsulated and spore-like in character, which, when conditions were favorable, multiplied and produced the forms usually observed in malarial infection. Celli believed that forms resulting from sexual multiplication remain inert in the viscera, such as the spleen and bone-marrow, and, when conditions were favorable, invaded the blood and gave rise to normal schizogonic forms. The observations of Schaudinn[1] are of especial interest in the consideration of this question. In his study of Plasmodium vivax he concluded that recurrences in malaria are due to parthenogenesis of the macrogametes which are not fertilized by the microgametes; these, remaining in man, eventually liberate schizonts which penetrate the erythrocytes, undergo sporulation, and produce the relapse. Schaudinn observed this process only in one case and describes it as consisting in the division of the chromatin of the nucleus, the death of a portion of the nucleus and protoplasm, and the segmentation of the remainder into forms which undergo schizogony. The forms undergoing parthenogenesis resemble very closely the forms resulting from intracorpuscular conjugation, so far as I have been able to follow them, and I believe that there is good reason for believing that at least some of the forms described by Schaudinn were in truth the large pigmented forms which result from this process.

Schaudinn claimed that parthenogenesis was complete in from nine to twelve days, and authorities, such as Ziemann and Mariotti-Bianchi, call attention to the occurrence of relaspes most frequently at this period of time. While relapses often do occur at intervals

[1] *Loc. cit.*

of nine to twelve days, it has not been my experience that the majority do, and it is very difficult, upon Schaudinn's assumption, to explain the occurrence of relapses occurring all the way from nine to eighty days after the initial attack, and the occurrence of latent infections, in which for weeks a few plasmodia may be demonstrated in the peripheral blood at intervals. I have observed relapses occurring at intervals of from two to four weeks very frequently, and several cases in which two to three months intervened between the malarial attacks. Taking these facts into consideration, I believe that it is much more rational to consider that the cause of relapse is some form of the parasite which is resistant to quinine, and which remains latent in the spleen or bone-marrow until conditions are favorable for development, when it undergoes mutiplication, and eventually develops into the forms which are familiar to us as schizonts; and I believe that it is the function of intracorpuscular conjugation to produce such forms of the plasmodia.

As is well known, rapid multiplication of certain protozoa leads to exhaustion and it is at this time in the life-history of the organisms that conjugation occurs, followed by a resting stage in some parasites, and subsequent multiplication when conditions are favorable for growth. It is significant that intracorpuscular conjugation occurs only in malarial patients who have suffered from repeated paroxysms; in other words, only in those cases in which rapid multiplication of the plasmodia has occurred for some time, in the usual manner. It is also more frequent in those cases in which quinine has been given in small and inefficient doses, and this again indicates that the process is one that develops only when conditions arise which are unfavorable to development in the normal manner. That the forms concerned in this process are resistant to quinine is another proof that they are intended to keep the infection alive within man.

The discontinuance of quinine in malarial patients is often followed by recurrences, even in patients who have been under treatment for a long time, and it is evident that during this time the forms of the plasmodia present in the body must have been resistant to the drug. Now, as a matter of fact, the only forms of the malarial plasmodia which resist quinine are the fully developed gametes and the forms concerned in intracorpuscular conjugation, and while the partheno-

genesis of the macrogamete might explain the occurrence of such relapses, *if macrogametes were always present*, it is a fact that relapses occur in many patients in whom the initial infection has not resulted in the development of any form of gamete. Thus, in a very large proportion of estivo-autumnal patients, *relapses occurred repeatedly but the gametes (crescents) were never observed in either the peripheral blood or in blood obtained by splenic puncture.* In such cases, which were numerous, how can parthenogenesis of the macrogametes explain the occurrence of the relapses? Intracorpuscular conjugation is observed very frequently in patients in whom no gametes can be demonstrated, and these facts are sufficient proof, to my mind, that latency and recurrence in the malarial fevers cannot be explained by the parthenogenesis of the macrogamete, and that in intracorpuscular conjugation we probably possess the true explanation of these phenomena.

After a certain period of rapid multiplication, or when the environment becomes unfavorable for schizogony in the usual manner, the process of intracorpuscular conjugation occurs, resulting in the development of a form of the plasmodium which is resistant to quinine and other injurious influences, and which, in all probability, becomes encysted and retires to the spleen or bone-marrow, where it remains in a resting condition until the environment again becomes favorable, when it liberates the spores which have developed within it, and these, penetrating the erythrocytes, develop eventually into schizonts. While it must be confessed that all of the steps in this process have not been witnessed, it is a fact that every step has been demonstrated except the morphology of the organism in the resting stage in the spleen or bone-marrow, and the actual sporulation of the large pigmented bodies resulting from the conjugation. Further research, especially in pernicious malarial infections, should result in additions to our knowledge of the exact form in which the plasmodia exist in the internal organs, and will, I am sure, support the theory that intracorpuscular conjugation is the cause of latency and recurrence.

The administration of quinine to a patient in whom this form of conjugation is occurring, is followed by the disappearance of the conjugating plasmodia from the peripheral blood, but this does not

prove that they are destroyed, but rather that they are driven by the presence of the drug to the internal organs. If the drug be administered before conjugation has begun, this process is never observed, and this is a strong argument in favor of the theory that this form of conjugation is the cause of recurrence, for it is well known that initial attacks of malaria, thoroughly and properly treated with quinine, do not suffer from relapses; while those that have been treated improperly, or in whom the infection has been present for several days before treatment, almost invariably relapse sooner or later.

In conclusion I would say that the importance of the subject of intracorpuscular conjugation, aside from its purely biological interest, lies in its relation to prophylaxis, for it is evident that if these forms of the plasmodia are the cause of relapse, it is essential that treatment be instituted promptly, and therefore, that it is necessary that the infection be recognized as early as possible. The early recognition of malarial infection depends upon the microscopical examination of the blood and this should never be neglected in any patient suffering from fever. The prophylaxis of malaria, especially in regions in which these infections are comparatively rare, often rests entirely in the hands of the attending physician, for the early recognition of the disease and the prompt institution of proper treatment will effectually put a stop to its spread, so far as that patient is concerned; while a false diagnosis, or improper treatment, will result in the patient becoming a source of infection to the community in which he resides.

EXPLANATION OF FIGURES A TO P.

The figures are diagrammatic and are intended to illustrate the phases of intracorpuscular conjugation observed in the blood in infections with Plasmodium vivax.

FIG. A.—Diagram of red blood corpuscle containing a "ring-form" of Plasmodium vivax: *A*, Chromatin of the nucleus of the plasmodium; *B*, Achromatic substance of the nucleus; *C*, Protoplasm of the plasmodium; *D*, Protoplasm of the red blood corpuscle.

FIG. B.—False conjugation. The two "ring-forms" are in apposition, the point of contact involving the chromatin only.

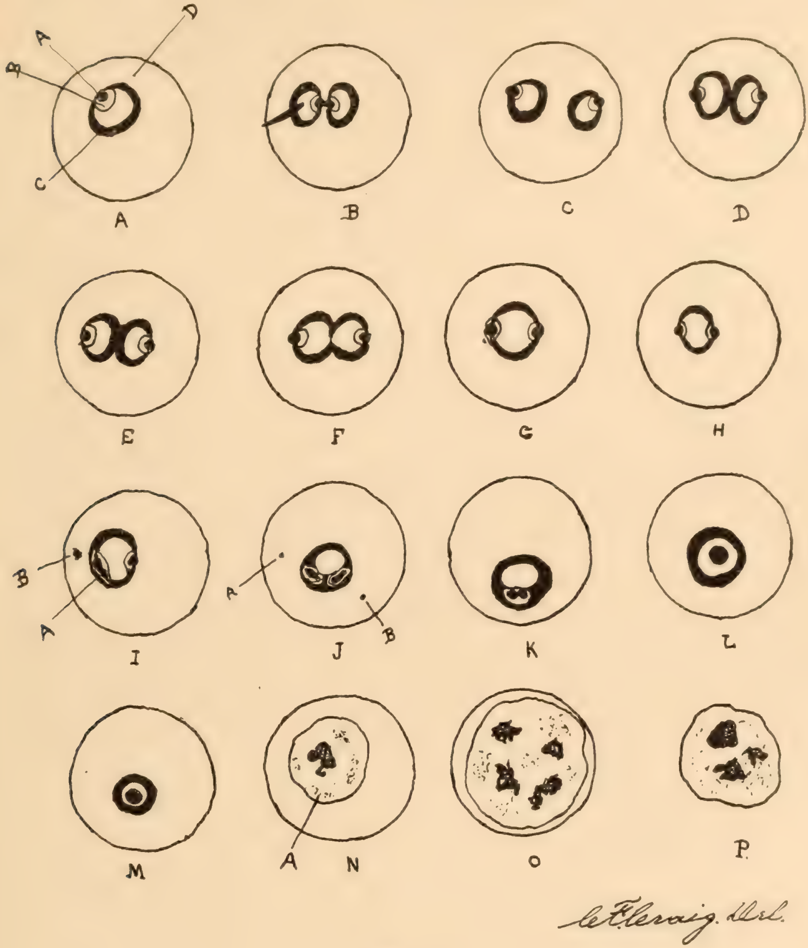
FIG. C.—Red blood corpuscle containing two "ring-forms" of Plasmodium vivax. No evidence of conjugation.

FIG. D.—First stage of conjugation. The "ring-forms" are in contact, the protoplasm alone being involved.

FIGS. E and F.—Illustrating the gradual blending of the protoplasm of the two organisms.

FIG. G.—Complete protoplasmic union of the two parasites. By this union one "ring-form" results, containing two masses of chromatin.

FIG. H.—Same as Fig. G but showing a smaller parasite.



FIGS. A–P.

FIG. I.—Conjugating organism in which the chromatin at *A* is lengthening and approaching the second mass of chromatin. At *B* a small particle of chromatin is seen which has been extruded from the conjugating organism.

FIG. J.—Conjugation form showing the approach of the two masses of nuclear chromatin. Note the lengthening of the chromatin masses. At *A* and *B* are observed extruded particles of chromatin.

FIG. K.—Conjugation form showing the union of the two chromatin masses, so

that the organism now consists of one large "ring-form," containing a single large chromatin mass.

FIGS. L and M.—Forms resulting after conjugation is completed. The organism consists of a mass of protoplasm, containing a large chromatin mass surrounded by considerable achromatic substance. These forms, except for their large size, resemble closely the tertian gametes in their earliest stages.

FIGS. N, O, and P.—The large pigmented forms which develop from the conjugating forms. Note the small amount of pigment (*A*).

ON THE DISTRIBUTION OF ANTIBODIES AND THEIR FORMATION BY THE BLOOD.*

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In order to study the distribution of antibodies in immunized animals, as well as certain other questions, dogs were injected intravenously with the blood of white rats or of goats. Only one injection was made, the amount injected being 1 c.c. of a 10 per cent suspension of the whole blood per kilo of dog. The suspension was made in m/8 NaCl solution containing one per cent of sodium citrate. The animals so injected were killed at different intervals after the injection and the amounts of antibodies in the blood, lymph, etc., determined. In this way estimations were secured of the antibody content in the fluids in question at different periods of immunization.

Collection of the fluids and methods of testing.—The lymph and aqueous humor were collected under ether anesthesia. The aqueous humor was drawn into a pipette forced through the cornea. Sterile glass cannulae were inserted in the neck lymphatics and the thoracic duct, and the lymph allowed to flow into sterile tubes. In the case of the neck lymph gentle massage of the head and neck was usually employed to hasten the flow. The blood was collected at the end of the collection of lymph. The cerebrospinal fluid was obtained through an opening into the fourth ventricle after the animal had been bled to death, this guarding against admixture with blood. The neck lymph always appears to be free from red corpuscles except when dissection is made cephalad to the point of insertion of the cannula in the lymphatic and in case of tearing of the skin and injury to the deeper parts of the head and neck as in fighting. The thoracic lymph may contain some erythrocytes even when there is no evidence of injury to the viscera, the trunk, or the legs. Perhaps the struggling of the animal during the anesthetization is responsible for the escape of red corpuscles into the thoracic lymph. In all cases the fluids from each animal, or set of animals, were treated in exactly the same manner; the blood and lymph were allowed to clot and the sera withdrawn after 18–24 hours; the fluids were tested at the same time under the same conditions and on the same objects. If it were necessary to keep the fluids for some time before testing them they were placed in a cold chamber at -1 or $-2°$ C. In almost every case the same identical experiment was made on two dogs of about the same size and weight which were kept under the same conditions. In the case of the dogs injected with rat blood all determinations were made with fresh, unheated fluids. In the case of the dogs injected with goat blood the lysin and opsonin determinations were made with unheated fluids. On account of prompt lysis of goat corpuscles by the lower dilutions

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of some of the fluids it was necessary to heat the fluids to 58° C. for 30 minutes for the agglutinin determinations. It was found that the maximum lysis by the antigoat fluids is obtained by the addition in each case of 0.012 c.c. fresh guinea-pig serum which in that quantity is without lytic power by itself. In Table 1, giving the results in question under "Lysin," the figures below the short line represent the highest dilution of unheated serum of fluid giving distinct lysis without the addition of 0.012 c.c. of guinea-pig serum, while the figures above the line give the highest dilution causing lysis with the addition of 0.012 c.c. of fresh guinea-pig serum.

In making these determinations the total quantity in each mixture was 0.6 c.c. Every mixture contained 0.2 c.c. of a five per cent suspension of washed, fresh corpuscles, goat or rat as the case might be; in the phagocytic experiments the remainder was made up of 0.2 c.c. suspension of dog leukocytes (obtained from pleural aleuronat exudates) and the antifluid tested plus the requisite amount of m/8 NaCl solution; and in the lytic and agglutinating experiments the remainder was made up by the fluid tested, 0.12 c.c. guinea-pig serum in the case of one of the lytic tests with goat corpuscles, and sufficient NaCl solution. After the usual incubation periods the lytic and agglutinating mixtures were placed in the ice-box for 18–24 hours when the results were determined, while in the case of the opsonic tests smears were made after an incubation of 60 minutes, and then stained. The results are given in Tables 1 and 2, the figures of which indicate the highest dilutions at which undoubted antibody action was observed on part of the fluids there mentioned. On Charts 1, 2, 3, 4, and 5 some of the results of the estimations of antibody content in the fluids of one dog of each set are plotted out in form of curves, the abscissae marking the day after injection on which the animals were killed and the fluids obtained, while the ordinates indicate the limits of antibody action. The results charted are those indicated by bold-faced type in Tables 1 and 2.

DISTRIBUTION OF SPECIFIC ANTIBODIES IN DOGS INJECTED WITH GOAT BLOOD.

The results shown in Table 1 and Charts 1, 2, and 3 indicate clearly that so far as the blood, the lymph from the thoracic duct, and the neck lymph are concerned, the changes in the concentration of antibodies during the course of active immunization run practically parallel. The concentration in the two lymphs is nearly the same, being on the whole a little less in the neck than the thoracic lymph, but in both it is always somewhat lower than in the blood-serum. In lymph as well as blood the antibody curves correspond quite accurately with the simple antibody curve obtained by determinations at short intervals of the content in antibody of the blood of the same animal. Indeed the general outlines of our composite curves correspond notably well with those of the simple antibody curve of blood-serum, especially when it is considered that the fluids from the different sets of animals were tested at different times, each time with

TABLE 1.

SPECIFIC ANTIBODIES IN BLOOD, LYMPH, AQUEOUS HUMOR, AND CEREBROSPINAL FLUID OF DOGS INJECTED WITH GOAT BLOOD.

LYSIN.

Days	Cerebrospinal Fluid		Aqueous Humor		Thoracic Lymph		Neck Lymph		Blood Serum	
1	0	0	..	..	**24**	24	**24**	12	**48**/48	../48
2	0/0	0/0	0/0	0/0	**12**/12	...	**24**/6	6/3	**48**/24	24/12
4	0/0	0/0	0/0	0/0	**384**/24	192/24	**384**/24	96/12	**1,536**/24	768/12
6	12/0	../0	0/..	..	**6,144**/12	../24	**3,072**/3	../12	**24,576**/24	../24
9	0/3	6/6	24/0	48/12	**3,072**/12	3,072/24	**384**/12	384/12	**12,288**/24	12,288/48
15	6/0	3/0	96/12	192/0	**768**/24	384/24	**1,536**/12	768/12	**12,288**/48	6,144/48
20	0/0	3/0	6/0	6/0	**48**/48	72/48	**192**/12	192/12	**768**/48	1,536/36
30	0/0	0/0	0/0	0/0	**96**/24	768/24	**192**/24	768/24	**768**/48	3,072/48
40	..	0/0	..	16/4	....	384/48	....	288/24	....	768/96

AGGLUTININ.

Days	Cerebrospinal Fluid		Aqueous Humor		Thoracic Lymph		Neck Lymph		Blood Serum	
1	0	0	..	..	**0**	0	**0**	0	**16**	8
2	0	0	0	0	**0**	..	**0**	0	**0**	0
4	0	0	0	0	**6**	6	**6**	12	**48**	48
6	0	0	..	..	**192**	192	**384**	192	**1,536**	1,536
9	0	0	0	0	**24**	48	**6**	24	**48**	96
15	0	0	0	0	**24**	24	**24**	12	**48**	48
20	0	0	0	0	**0**	0	**0**	0	**24**	24
30	0	0	0	0	..	..	..	..	..	..
40	..	0	..	0	..	12	..	12	..	24

OPSONIN.

Days	Cerebrospinal Fluid		Aqueous Humor		Thoracic Lymph		Neck Lymph		Blood Serum	
1	0	1	..	..	**12**	6	**6**	6	**12**	6
2	0	0	0	0	**6**	..	**6**	0	**0**	0
4	0	0	3	0	**24**	24	**6**	6	**36**	12
6	3	6	..	..	**192**	192	**192**	96	**768**	192
9	12	12	6	24	**92**	192	**48**	96	**96**	384
15	4	6	6	96	**384**	192	**96**	96	**768**	384
20	3	6	6	6	**86**	192	**48**	96	**96**	192
30	1	1	1	1	**48**	96	**48**	96	**96**	192
40	..	0	..	0	..	48	..	48	..	96

First day=normal dogs. Bold-faced figures=see Charts 1, 2, 3.

0=no action in dilutions of 1 to 1 or 1 to 3.

Under "Lysin" figures under short line give highest dilution with lytic power of the fluid in question without addition of guinea-pig serum; figures above the line represent highest dilution with lytic power in presence of 0.012 c.c. fresh guinea-pig serum.

a different suspension of goat corpuscles, and in the phagocytic experiments consequently also with different suspensions of leuko-

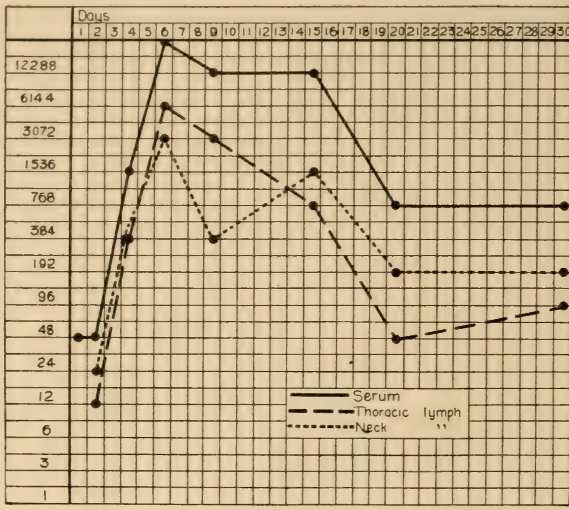

CHART 1.—Specific lysin in serum and lymph of dogs injected with goat blood.

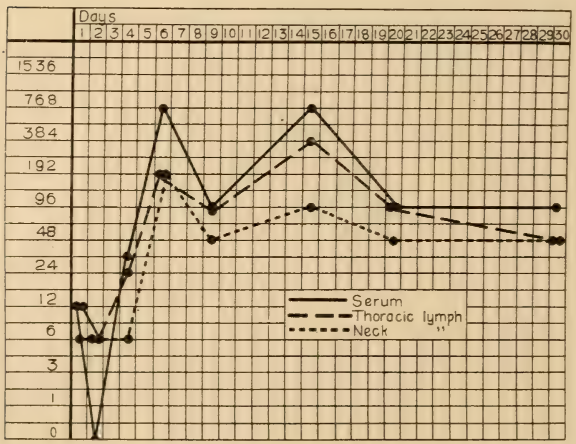

CHART 2.—Specific opsonin in serum and lymph of dogs injected with goat blood.

cytes as well. This last, together with variations in the power of individual animals to produce antibodies (compare the results obtained

in the two dogs of the same set, Table 1), no doubt in large measure accounts for the irregularities in the curves.

We may say, then, that in dogs injected with goat blood the newly formed antibodies in the lymph appear and disappear at the same

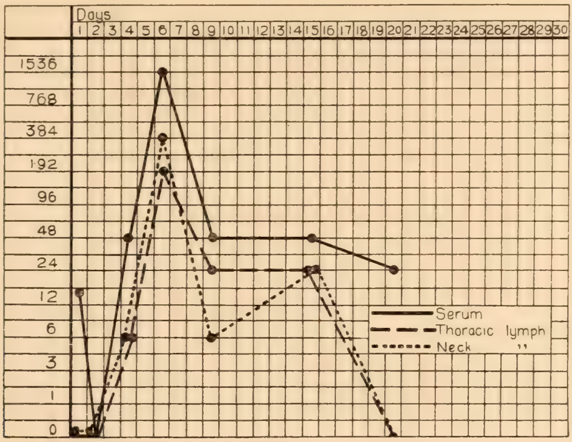

CHART 3.—Specific agglutinin in serum and lymph of dogs injected with goat blood.

time and describe the same wavelike curve as those in the blood. The relative concentration of the antibodies in the blood and in the lymph appears to be quite constant, but it would require further and more accurate measurements to determine the exact quantitative relations that obtain under various conditions.

As regards the cerebrospinal fluid and aqueous humor we note in the first place the complete absence in these fluids at any stage of the reaction of agglutinin. Lysin and opsonin, however, were present in both the fluids during the period of high antibody content in the blood and lymph (Table 1), but only in minute traces.

DISTRIBUTION OF SPECIFIC ANTIBODIES IN DOGS INJECTED WITH RAT BLOOD.

The results given in Table 2 and Charts 4 and 5 were all obtained with fresh serum. We note first of all the failure to obtain any evidence whatsoever of any increase in the lysin in the injected dogs. Thinking that perhaps owing to deficient or unsuitable complement

the absence of new lysin was merely apparent and not real, we made numerous trials with the fresh sera of the different laboratory animals as complement for antirat dog serum, and heated immune serum

TABLE 2.

SPECIFIC ANTIBODIES IN BLOOD, LYMPH, AND CEREBROSPINAL FLUID OF DOGS INJECTED WITH RAT BLOOD.

LYSIN.

Days	Cerebrospinal Fluid		Thoracic Lymph		Neck Lymph		Blood Serum	
1	0	0	6	6	6	6	12	12
2	0	0	3	0	3	0	12	12
3	0	0	1	1	1	1	12	12
6	0	0	6	6	3	3	12	12
9	0	0	6	12	0	3	12	12
12	0	0	12	6	0	3	12	12
16	0	0	6	..	1	1	12	12
20	0	0	9	9	3	3	12	12
30	0	..	6	..	24	..	12	..
44	0	..	6	..	6	..	12	..

AGGLUTININ.

Days	Cerebrospinal Fluid		Thoracic Lymph		Neck Lymph		Blood Serum	
0	0	0	**48**	48	**48**	48	**96**	96
1	0	0	**3**	0	**3**	0	**24**	24
3	0	0	**12**	12	**24**	12	**80**	80
6	0	0	**192**	96	**192**	72	**384**	384
9	0	0	**384**	768	**384**	384	**3,072**	6,144
12	0	0	**384**	192	**384**	192	**1,536**	1,536
16	0	0	**192**	192	**192**	192	**1,536**	1,536
20	0	0	**768**	768	**768**	768	**1,536**	1,536
30	0	..	**384**	...	**192**	...	**1,536**	....
44	0	..	**72**	...	**0***	...	**192**	....

OPSONIN.

Days	Cerebrospinal Fluid		Thoracic Lymph		Neck Lymph		Blood Serum	
1	**3**	3	**24**	24	**24**	24	**48**	48
2	3	3	**3**	3	**3**	3	**28**	24
3	0	0	**0**	24	**12**	24	**36**	36
6	6	12	**192**	192	**192**	192	**1,152**	1,152
9	48	48	**576**	768	**768**	768	**3,072**	6,144
12	192	192	**768**	768	**768**	768	**3,072**	3,072
16	24	6	**384**	384	**384**	384	**1,536**	768
20	12	12	**768**	768	**768**	768	**1,152**	1,152
30	12	..	**192**	...	**96**	...	**1,536**	....
44	12	..	**48**	...	**0***	...	**192**	....

* Lymph from hyperplastic goiter.

First day=normal dogs. 0=no action in dilutions of 1 to 1 or 1 to 3. Bold-faced figures=see charts 4 and 5.

plus fresh normal dog serum was used in varying proportions, but always with negative results. In the case of certain other dogs immunized with rat corpuscles a comparatively slight increase in lysin was observed, by use of either fresh serum or heated serum complemented with guinea-pig serum, while in a splenectomized dog

injected with rat corpuscles no increase in lysin was demonstrated.[1] It is possible, of course, that further search may reveal a method or

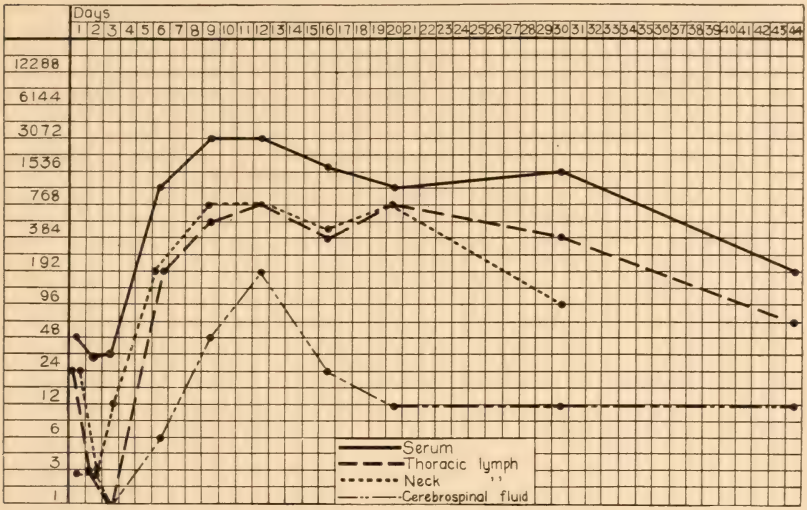

CHART 4.—Specific opsonin in blood, lymph, and cerebrospinal fluid of dogs injected with rat blood.

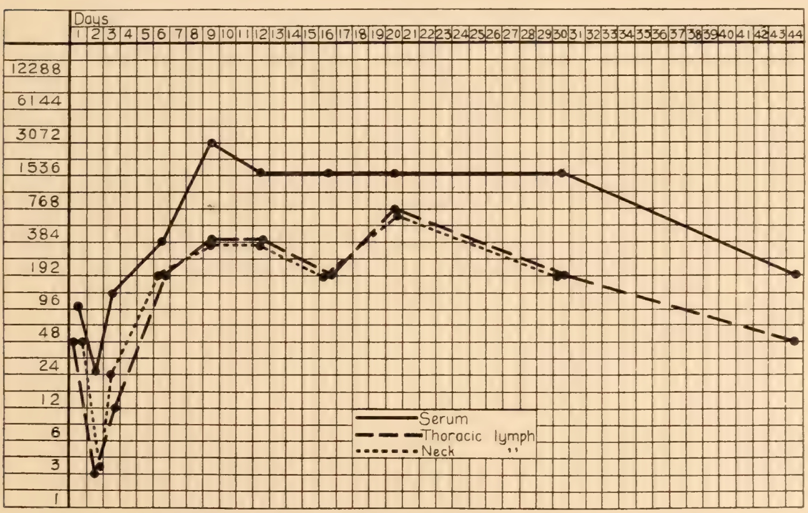

CHART 5.—Specific agglutinin in blood and lymph of dogs injected with rat blood.

methods that will bring to light an increase in lysin in dogs injected with rat corpuscles; the fact remains that with the methods we used

[1] Hektoen, "Opsonins Distinct from Other Antibodies," *Jour. Infect. Dis.*, 1909, 5, p. 78.

no increase was demonstrable in a single animal of the whole series. The agglutinin and the opsonin for rat corpuscles as compared with each other occur in about the same amount in the blood-serum, the thoracic lymph, and the lymph from the neck (Table 2 and Charts 4 and 5). Here again the blood-serum contains more antibodies than the two kinds of lymph; in the latter the concentration is about the same; the relative concentration in the blood-serum and the lymph is fairly constant, and the composite curves correspond closely with the normal or typical, simple antibody curve of the blood, a distinct negative phase being present in every curve. Finally, it is especially noteworthy that opsonin only was demonstrable in the cerebrospinal fluid in which it gives a fairly typical antibody curve; this would seem to indicate that this particular antibody finds an easy passage into the fluid from the blood and lymph, possibly with the water stream.

DISTRIBUTION OF THE SPECIFIC ANTIBODIES IN DOGS TRANSFUSED WITH THE BLOOD OF DOGS INJECTED WITH GOAT BLOOD.

In connection with certain other experiments it seemed desirable to investigate the occurrence and distribution of specific antibodies in dogs which had been transfused freely from dogs injected with goat blood. It was hoped that some light might be thrown on the question of the production of antibodies by leukocytes or other elements in the circulating blood and on the relative distribution of antibodies in the passively immunized animal.

As in the other experiments the immunization of the donors was effected by the intravenous injection of 1 c.c. of a 10 per cent suspension of goat blood per kilo of dog.

Transfusion.—In most of the transfusion experiments the donors were large dogs, weighing 22–28 K, and the recipients small animals, weighing 4–8 K. Hence one large dog would serve as donor for two of the smaller ones in transfusion from artery to artery and in cases of artery-vein transfusion, which was made in the usual way, one large dog would serve as donor for three small ones, because by the latter method the blood encounters no resistance in the recipient.

In the experiments involving the collection of lymph from the recipients, it was necessary to transfuse from artery to artery in order not to occlude any veins because venous occlusion and passive hyperemia quickly affect the character of the lymph from the region involved. With large dogs as donors and small dogs as receivers this result is attained by connecting the distal end of the carotid of the recipient with the

proximal end of the opposite carotid of the donor. This involves ligation of one carotid in the recipient which does not seriously alter the circulation in the neck and head. A "T" cannula of glass, coated on the inside with oil or paraffin, was used to connect the arteries of the two dogs. Theoretically a Crile cannula is superior to this device, but as thrombosis is equally well avoidable, the simple glass cannula was preferred because it may be manipulated more quickly. Placing the bulldog forceps on the carotids of both dogs proximally to the insertion of the cannula and carefully removing the blood in the free ends of the arteries before inserting the cannula prevents the blood from coming in contact with the cannula except during the transfusion and this contact does not start clotting. Donor and recipient, both under ether anesthesia, are placed on the back and the left carotid in one and the right carotid in the other is isolated and severed; the necks of the dogs are then brought closely enough together to permit the insertion of a "T" cannula in the proximal end of the carotid of the donor and in the distal end of the carotid of the recipient. The cannula is then filled with Ringer's solution and all air bubbles carefully removed. Everything being ready for the transfer of blood the recipient is bled "dry" from the proximal end of the severed carotid, that is, the carotid is left open until the flow of blood from it ceases; at this point no pulse is discernible anywhere, altho a feeble cardiac impulse may still be detected; the respiration is feeble, slow, and irregular, or it may have ceased. The clamp on the carotid of the donor is now removed and the donor pumps his blood into the empty vessels of the recipient until the pulse and blood pressure of the latter are brought up to the normal. In most of our experiments the transfusion was continued until the recipient's pressure was slightly higher than before the bleeding. The carotids of the recipient are now tied and the wound closed. The animals recover quickly from the anesthesia and in no case so far have any untoward symptoms ascribable to the exchange of blood developed.

Obviously the recipient loses the greater part of his own blood in this procedure and receives approximately an equal amount from the donor. Of course we are not dealing with exact quantities. The only criterion of the quantity of blood transferred from the donor is the blood pressure of the recipient which to a great extent depends on the condition of the vasomotor centers. It is probable that the normal activity of these centers is disturbed more or less by the temporary anemia of the nervous system and the collapse of the vessels. In nearly all our experiments the large dogs acting as donors were killed at the end of the transfusion. The collection of fluids and the estimation of antibodies were carried out in the manner already described.

The results of the experiments on the distribution of antibodies in the body fluids of dogs after bleeding and transfusion with immune blood may be disposed of quickly. As the figures in Table 3 show, the antibodies introduced in this manner very soon pass into the thoracic and neck lymph—one-half to three hours—and maintain about the same proportion in these fluids relative to that in the blood as in the actively immunized animals. The indications are that the antibodies appear in the thoracic lymph first, at least under certain circumstances, but further experiments with blood richer in anti-

bodies than used in our earliest determinations after transfusion are necessary to settle such questions. The results fail to show any notable difference in the rate of passage of the different antibodies from the blood to the lymph, but smaller differences might not be

TABLE 3.

ANTIBODIES IN FLUIDS OF DOGS TRANSFUSED WITH BLOOD OF DOGS INJECTED WITH GOAT BLOOD.

FLUIDS	HOURS AFTER TRANSFUSION WHEN FLUIDS WERE COLLECTED								
	30 Min.			3 Hours			24 Hours		
	Lysis	Aggl.	Ops.	Lysis	Aggl.	Ops.	Lysis	Aggl.	Ops.
Blood-serum	1,536	96	24	12,288	348	348	3,072	192	
Thoracic lymph	192	24	12	3,072	192	48	768	96	24
Neck lymph	12	0 (12)	0 (12)	768	24	12	384	48	12
Cerebrospinal fluid	3	0 (3)	0 (3)	12	0 (6)	0 (6)	6	0 (6)	0 (6)
Aqueous humor	12	3	0 (3)	24	6	8	0(6)	0 (6)	0 (6)

The figures in parentheses mean that there was no action in the dilution expressed by the figure.
Concentration of antibodies in blood-serum of donors at time of transfusion:

½ hour —lysis 6,144, agglutinin 384, opsonin 192
3 hours— " 24,576, " 1,536, " 1,536
24 hours— " 3,072, " 384, " 192

demonstrated by the method of dilution we used. Our figures indicate that in transfused animals traces of antibodies early may find their way into the cerebrospinal fluid and aqueous humor, but for unknown reasons this does not appear to take place constantly. Undoubtedly the rapid passage of antibodies from the blood into the lymph serves to explain to some extent the prompt early loss by blood in antibody in passive immunization.[1]

The fact that the relative distribution of antibodies on passive immunization is essentially the same as on active immunization indicates that the distribution in the latter case is determined by the rate of passage of antibodies from the blood to the lymph rather than by their place of formation. This conclusion seems well founded because the relative concentration is practically the same at all points of the immunization curves.

The number of instances in normal as well as immunized animals in which various antibodies have been found more concentrated in the blood than in the lymph and also more concentrated in the thoracic

[1] Levin, "Ueber passive Immunität," *Ztschr. f. Immunitätst. u. exp. Therapie*, 1909, 1, p. 3.

than in the neck lymph[1] makes it highly probable that this is the rule with respect to the concentration in these fluids of antibodies in general especially in the blood and lymph. In connection with this generalization it is interesting to note that Carlson and Luckhardt[2] find that the diastases in the normal body fluids are similarly distributed. It might be interesting to determine further the relative concentration of antibodies in the case of animals subjected to forced immunization.

THE EFFECT OF TRANSFUSION OF BLOOD OF DOGS IN THE LATENT PHASE OF ANTIBODY FORMATION—ARE ANTIBODIES FORMED IN THE BLOOD?

We now invite attention to Table 4 which gives the results of estimations of antibodies in the blood of dogs transfused from dogs at

TABLE 4.

ANTIBODIES IN THE SERUM OF DOGS TRANSFUSED WITH IMMUNE BLOOD AT VARIOUS PERIODS—3, 6, AND 24 HOURS, AND 2, 4, AND 6 DAYS—AFTER INJECTION OF DONORS WITH GOAT BLOOD (1 C.C. 10 PER CENT SUSPENSION PER K).

Days after Transfusion	No. of Hours and Days after Injection of Donor with Antigen									
	3 Hrs.	6 Hrs.	24 Hrs.	48 Hrs.	4 Days			6 Days		
	Lysin	Lysin	Lysin	Lysin	Lysin	Aggl.	Ops.	Lysin	Aggl.	Ops.
Just before transfusion.........	24	24	24	16	32	8	8	24	12	12
Just after transfusion.........	24	24	24	16	768	48	24	6,144	192	192
2...............	24	24	24	..	...	..	..	...	...	...
3...............	..	..	24	24	192	24	24	3,072	96	96
4...............	24	24	24	..	...	..	..	....	..	..
5...............	..	..	48	..	...	..	..	...	..	..
6...............	24	24	48	..	...	..	..	...	..	..
7...............	24	24	24	12	144	16	8	384	48	48
8...............	24	24	24	..	...	..	..	...	..	..
10...............	24	24	..	..	...	..	..	...	..	..
11...............	..	..	24	..	...	..	..	...	..	..
14...............	..	..	..	..	48	16	8	48	24	24

various periods after the injection of goat blood. One outstanding feature is that no antibodies appear to have been produced by or in the transfused blood itself or as a consequence of the transfusion. This is indicated especially by the absence of any increase of antibodies

[1] Braude and Carlson. "The Influence of Various Lymphagogues on the Relative Concentration of Bacterioagglutinins in Serum and Lymph," *Amer. Jour. Phys.*, 1908, 21, p. 221; Hughes and Carlson, "The Relative Hemolytic Power of Serum and Lymph under Varying Conditions of Lymph Formation," *ibid.*, p. 237; Becht and Greer, "A Study of the Concentration of the Antibodies in the Body Fluids of Normal and Immune Animals," *Jour. Infect. Dis.*, 1910, 7, p. 127.

[2] "On the Diastases in the Blood and Body Fluids," *Amer. Jour. Phys.*, 1908, 23, p. 148.

in the animals transfused from donors in an earlier stage of immunization than the fourth day. If antibodies are formed in or by the blood of immunized animals it would be reasonable to look for a production of antibodies in the receivers of the blood of dogs injected with goat blood two days and earlier before the transfusion, especially in view of the large amount of blood successfully transfused. But no increase took place.

The diminution of antibodies in the recipients of blood that contained newly formed antibodies follows the course typical of passive immunization as established by Madsen[1] and others, the decrease at first taking place rather rapidly and then more slowly, as shown well by the determination in the case of transfusion on the eighth day after the donor was injected with goat blood and when the concentration of antibodies was at its height.

Our results consequently warrant the inference that in suitable animals antigens are quickly removed from the blood or in some way so changed that the antigenic property is lost. At all events we may say that in dogs injected intravenously with 1 c.c. 10 per cent suspension of goat blood per kilo of weight, which seems to be an optimum antigenic dose in this particular case, this removal or change of antigen takes place within 3–48 hours; for there is no new formation of antibodies in dogs transfused with a large part of the blood of dogs so injected. If the blood so transfused contained free and unchanged antigen, antibodies surely would have developed in the recipient. In full concord with this result as to the recipient is the fact that the new formation of antibodies proceeds in a perfectly typical manner in donors that are transfused immediately from healthy dogs, the curve in some cases reaching a very high mark, possibly on account of the stimulus of the loss of blood on the blood-forming organs which we have reason to believe play a principal part in the formation of antibodies.

It is reasonable to assume that the power of the antibody forming cells to take up antigenic substances sooner or later reaches its limit. If that be the case the quantity of antigen in excess of this limit might remain in the circulating blood for some time after its introduction. After the injection of rabbits with 30–35 c.c. of ox blood freed from

[1] Th. Madsen, *The Decrease of Antibodies in the Organism Indicated by a Formula* (Festskrift, Statens Serum Institut, Copenhagen, 1902).

serum, Sachs[1] found by means of a specific lysin which did not lake rabbit corpuscles, that ox blood remained free in the rabbit blood for two to three days and even longer, disappearing in a more or less critical fashion as the lytic amboceptor was produced in response to the immunization. Free excess of antigen may be detected also by the method of transfusion as shown by the following experiment:

The donor was injected with 1 c.c. of goat blood per kilo of weight 14 hours previously; just before the transfusion 500 c.c. of blood were removed from the recipient; the transfusion was continued until the pressure reached normal; the 500 c.c. of blood removed from the recipient were defibrinated and infused into the donor. The following figures give the highest lytically active dilution of the serum of the recipient in the presence of 0.2 c.c. of a 5 per cent suspension of goat corpuscles and 0.012 c.c. of fresh guinea-pig serum:

Days after Transfusion	Highest Active Dilution of Serum
1	24
2	24
3	48
4	48
6	768
8	1,536
10	1,536
12	768
14	384
17	384
19	192
21	192

So far as the amount of lysin in the blood of the recipient indicates, a small quantity only of free antigen was introduced in the transfused blood. Evidently the larger part of the goat blood had been removed from the blood of the donor, and that the antibody-forming cells took part in this removal is indicated by the fact that antibody formation proceeded without disturbance, the serum on the sixth day causing lysis in a dilution of 1:36,864.

Our results consequently support the view that antibodies in active immunization are produced outside of the circulating blood into which they find their way at the end of the latent period.[2]

[1] Sachs, "Ueber die Vorgänge im Organismus bei der Transfusion fremdartigen Blutes," *Arch. f. Anat. u. Physiol.*, 1903, p. 44.

[2] V. Dungern (*Die Antikörper*, Jena, 1903) transfused normal rabbits from rabbits in the stage of latency in precipitin formation following the injection of crab plasma (Maja squinado). The transfused animals showed no sign of precipitin formation; they seemed, however, to respond to injection of crab plasma somewhat more quickly than fully normal animals, but whether because blood cells are concerned in antibody formation or for other reasons is not known.

SUMMARY.

In active immunization of dogs by a single intravenous injection of goat blood the lysin, agglutinin, and opsonin for goat corpuscles reach their highest concentration in the blood. They are uniformly slightly less concentrated in the thoracic lymph and the neck lymph, while in the cerebrospinal fluid and aqueous humor only traces of the lysin and the opsonin can be detected at the height of immunity.

In dogs similarly immunized with rat blood the opsonin and agglutinin in the blood and lymph describe parallel curves, the concentration being greatest in the blood. In the cerebrospinal fluid opsonin only is present and while the concentration is much lower than that of the blood and lymph, the curves are parallel. No increase in lysin for rat corpuscles was demonstrated by the methods used.

This relative concentration of antibodies in the blood and lymph seems to obtain in normal animals as well as in all the stages of immunization.

On transfusion of blood of dogs immunized with goat blood into normal dogs previously bled dry through the carotid the antibodies can be detected in the lymphs of the recipient in 30 minutes and in a short time the same relative distribution of the antibodies is effected as in active immunization. It seems therefore probable that in active immunization the distribution depends on the equilibrium relation between the blood and the lymph rather than on the place of formation of the antibodies, and that the rate of passage of the antibodies from the blood to the lymph is probably in part a function of the concentration in the blood.

There appears to be no difference in the rate of passage of the various antibodies for the blood to the lymph, but our methods might not disclose slight variations.

When the blood of an immune animal is transfused into a normal animal previously bled dry there is a rapid fall in the concentration of antibodies during the first 24 to 48 hours; then follows a more gradual disappearance of the antibodies until the normal limit is reached, the rate of diminution being a measure of the rate of destruction and elimination of antibodies as there is no production in the transfused blood. Hence the duration of passive immunity after as

complete transfusion as possible depends on the concentration of the antibodies in the donor's blood and the quantity of this blood transfused, a point of possible importance in connection with direct transfusion for therapeutic purposes in infectious diseases.

Bleeding a dog dry while in the latent period after intravenous injection of goat blood and then transfusing him from a normal dog may have no other than a stimulative effect on the processes of antibody formation. And transfusion of a normal dog (previously bled dry) from a dog injected intravenously with an optimum antigenic dose of goat blood from 3 to 48 hours previously, does not lead to the production of antibodies in the recipient. If the transfusion is made some time later than indicated after the introduction of the antigen the result is a simple passive immunization. These facts indicate that in this case the blood takes no direct part in the fixation of the antigen or the production of antibodies.

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THE RELATION OF THE PSEUDODIPHTHERIA AND THE DIPHTHERIA BACILLUS.*

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CONTENTS.

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INTRODUCTION.

WITH the advance of our knowledge of the bacteria, both pathogenic and non-pathogenic, numerous organisms have been discovered similar to the normal type in most respects, but differing from it in certain characters. Bacteria are extremely variable organisms, altho the limits to this variability seem to be more sharply defined than was formerly supposed. This variability depends largely upon two main factors. The first is the enormous number of generations which may develop in a short period of time. With the bacteria it is indeed true that "one day is as a thousand years." The second is the fact that because of the simple single-celled structure of bacteria, acquired characters can, to a certain extent, be inherited. The effect of the environment is, therefore, of great importance in any study of bacteria. Variations, caused by the nature of the conditions under which organisms have recently been living, must be considered as well as the ordinary fluctuating variations and mutations found in all forms of plants and animals. It is because of these facts that the idea of "groups" among the bacteria has developed in recent years. Within each group are found many different types or species which add greatly to the difficulties of identification and present puzzling problems as to the nature of the interrelationship among the several forms.

The group of diphtheria organisms exhibits, possibly more than any other, a greater variety of these somewhat similar types. Löffler himself discovered the so-called pseudodiphtheria bacillus in 1887 and a few months later it was independently observed by Hofmann-Wellenhof (1888). B. xerosis was described by Kutschbert and Neisser (1884) and since that time a large number of other somewhat similar types, known generally as diphtheroids, have added to the general interest and confusion. In this group, also, the problem of relationship is of special importance because of its bearing on the bacteriological control of contacts, carrier cases, quarantine, treatment of epidemics, and other public-health methods.

There have been, in general, two theories held by the different observers in regard to relationships of the different organisms of this group. On the one hand, we find such men as Roux, Yersin, Behring, Wesbrook, and others upholding the view that all of these types are

variants of the same species. On the other hand, we find by far the greater number of bacteriologists, including Löffler, Escherich, Fränkel, Flügge, etc., believing that at least the first three forms mentioned are three distinct species, while opinion seems to be about evenly divided as to the classification of the diphtheroids.

Particularly among the earlier writers, considerable confusion existed as to the exact meaning of the term pseudodiphtheria bacillus. The descriptions of both Hofmann and Löffler in regard to this organism are rather indefinite. There can be little doubt that many of the discrepancies apparent in the literature are due to the inaccuracy of our definitions and that many of the writers have been dealing with atypical Klebs-Löffler bacilli.

In later years, there has been a tendency to distinguish between the different organisms by the difference in pathogenicity and in their action in dextrose broth. Park and Beebe's (1895) classification based on these points, is as follows:

I. Bacilli identical in appearance, both in culture and under the microscope, with the diphtheria bacillus.
   a) Pathogenic acid producers equal virulent diphtheria bacilli.
   b) Non-pathogenic acid producers equal non-virulent bacilli.

II. Bacilli somewhat resembling, but shorter and stouter than diphtheria bacilli.
   a) Non-pathogenic, non-acid producers equal Hofmann's or the pseudodiphtheria bacilli.

The use of this scheme and also Wesbrook's more elaborate classification has given greater accuracy to the observations of the more recent workers in this field.

REVIEW AND DISCUSSION OF PREVIOUS WORK ON THE SUBJECT.

The distinctive features generally used for purposes of differentiation between the various members of this group are: 1. *Cultural characteristics*. 2. *Staining reactions*. 3. *Action upon carbohydrates*. 4. *Serum reactions*. 5. *Morphology*. 6. *Pathogenicity*.

The first two of these may be dismissed very briefly as practically all recent observers admit that, while these points are of service generally, they are not sufficiently constant to be made the criterion of specific difference. It is generally recognized that B. diphtheriae varies greatly on the different culture media and therefore no reliance can be placed upon that feature. Even the double stains of Neisser, Falieres, Ljubinsky, and others are unreliable, and many observers prefer the ordinary Löffler's stain which is simpler and gives exactly as much information as any of the more complicated processes.

The other characteristics are of more importance, however, and it may be well to review briefly some of the more recent researches upon these points.

Action upon carbohydrates.—Roux and Yersin (1888) appear to be the first to have noticed that the diphtheria bacillus produced acid in broth and most subsequent observers have agreed with them. Zarniko (1889) observed that Hofmann's bacillus produced in broth cultures either an alkaline reaction or at first a weakly acid followed by an alkaline reaction. Escherich (1894) went so far as to declare that acid production was proof positive of the pathogenicity of a given strain.

Spronck (1895) pointed out that if diphtheria organisms are grown in dextrose-free broth, the reaction remains alkaline; if small quantities of the muscle sugar are present, the reaction becomes at first acid and later alkaline; while if much dextrose is added, the broth attains an acid reaction which is permanent. Numerous observers have confirmed these results.

Less work has been done on the other sugars, but according to L. Martin (1898) the diphtheria bacillus produces acid from dextrose, levulose, galactose, saccharose, and glycerin, but not from maltose, lactose, mannit, arabinose, raffinose, dulcite, glycogen, erythrite, and starch. Practically all other writers differ with him, however, as regards the action on saccharose.

More recently Knapp (1904), in testing 27 cultures of diphtheria bacillus, 10 of xerosis bacillus, and 4 of pseudodiphtheria bacillus, came to the conclusion that the action in saccharose and dextrin media will differentiate between these three species, as follows:

The pseudodiphtheria bacillus ferments neither sugar.

The xerosis bacillus ferments saccharose.

The true Klebs-Löffler bacillus ferments dextrin.

Hamilton and Horton (1906) were unable to confirm Knapp's work except as regards dextrin.

Graham-Smith (1906) describes experiments on 23 cultures of diphtheria bacilli (18 fully virulent, 5 non-virulent), 20 cultures of Hofmann's bacilli, 3 cultures of xerosis bacilli, and some other diphtheria-like bacilli. He used mostly the serum medium of Hiss with an incubation of 10 days and concludes from this work that some of the contradictory results obtained by various investigators are due to the fact that many strains of organisms will not grow well in broth when first isolated from the throat. His tests show that under suitable conditions all strains of diphtheria bacilli produce acid from glucose, galactose, levulose, and maltose and the majority from dextrin and glycerin. The action on lactose is very variable and only a few strains act on saccharose. Hofmann's bacillus produced acid in no case. The xerosis bacillus produced a small amount of acid from glucose, levulose, and glycerin, and a still smaller amount from saccharose. Zinsser (1907) working with 42 strains of B. diphtheriae, 21 strains of B. xerosis, and 8 strains of B. hofmanni confirmed Knapp's results. Goodman (1908) examined the action of 103 strains of Klebs-Löffler bacilli on dextrose, dextrin, maltose, and saccharose. By titration, he found a wide range of variation in the amount of acid produced, that from dextrose extending from $+0.1$ to $+4.0$. From one strain, he made a series of artificial selections, choosing each time the highest and lowest acid producer. Starting with $+2.3$ and $+1.9$ as the high and low cultures, after 36 selections, he obtained $+4.4$ and -0.5 as the greatest and the least acid producers respectively. The morphology of these last two cultures was still similar to that of the parent culture. The pathogenicity, however, was not satisfactorily tested. He concludes that the division of the diphtheria group into several distinct species is probably based on a misconception.

Serum reactions.—Spronck (1896) describes a test in which he inoculates a guinea-pig with antitoxin and then with the organism to be examined. Complete protection against the organism denotes it to be the true diphtheria bacillus; local edema only, as in the controls, denotes the pseudodiphtheria bacillus. This test is, of course, valueless with wholly non-virulent organisms. C. Fränkel (1897) confirms these results. Bergey (1898) could produce no immunity against the Klebs-Löffler bacillus by several inoculations of Hofmann's bacillus. Lubowski (1900) immunized a goat by inoculating with non-virulent diphtheria bacilli. The serum thus obtained agglutinated 23 strains of typical diphtheria organisms and two of non-virulent organisms at a dilution of 1:80. B. hofmanni and cocci were not agglutinated.

Lesieur (1901) immunized a horse against Klebs-Löffler bacilli and obtained a serum which agglutinated some strains of diphtheria organisms but not others. It acted in a similar fashion toward the pseudodiphtheria bacilli. Fifty of his 70 strains were not agglutinated.

Schwoner (1902) obtained a serum by injecting a horse first with dead and then with living B. diphtheriae. This agglutinated diphtheria bacilli at a dilution of 1:500 and some strains as high as 1:10,000. It agglutinated pseudodiphtheria organisms at a dilution of 1:10 to 1:40 exactly as in the case of normal and antitoxic sera. He also (1904) shows that cultures of diphtheria bacilli from severe cases exhibit hemolytic power on rabbits' corpuscles. Pseudodiphtheria bacilli do not show this action.

Hamilton and Horton (1906) obtained a serum from goats and rabbits immunized against one strain of their virulent pseudodiphtheria bacilli. This proved to be bactericidal to the other virulent pseudodiphtheria strains but not to non-virulent pseudodiphtheria bacilli nor to true diphtheria bacilli.

Morphology.—Roux and Yersin (1890) discovered no morphological alteration of a strain of B. diphtheriae by growth at 39°5 C. Hewlett and Knight (1897) state that the pseudodiphtheria bacillus seems to replace the typical diphtheria organism during convalescence. They illustrate this with a list of bacteriological examinations of 24 cases. Wesbrook, Wilson, and McDaniel (1900) introduced a classification of the various types of diphtheria organisms based on an extensive study of the morphology in pure cultures. These types are divided into three main groups: granular, barred, and solid-staining forms with numerous subdivisions based upon size and shape. They observe that the granular types usually give place wholly or in part to barred and solid types shortly before the disappearance of diphtheria-like organisms.

Gorham (1901) corroborates this and believes that the change is caused by the effect of the body fluids of persons becoming slowly immune or those entirely non-susceptible. Lesieur (1901), by growth for eight months in diffuse daylight in a dry room, transformed three diphtheria strains to pseudodiphtheria types. By passage in collodion sacs through three rabbits, B. pseudodiphtheriae took on the morphology of B. diphtheriae. He was also able to do this with two out of four strains of the pseudodiphtheria bacillus by repeated transfer in broth, and in one out of three, by a single passage through broth in symbiosis with Aurococcus aureus.

Cobbett (1901) records a series of examinations made during the progress of the disease in a number of cases and came to the conclusion that D^2 (Wesbrook's classification) does not replace typical forms during convalescence. He suggests that a possible cause of observations to this effect might be that in the early stages of the disease the diphtheria organisms are readily found so that a more careful examination is omitted. Later, the diphtheria organisms become fewer and a more vigorous search

must be made. In the course of this more thorough examination, naturally more D^2 are met with and a false impression as to their relative prevalence is produced. He also states that D^2 is not more frequently found in the noses and throats of persons in close relation with those sick with diphtheria than in those who apparently have had no contact with the disease:

Ohlmacher (1902) changed a typical diphtheria organism to an organism resembling Hofmann's bacillus by 48 hours' subcutaneous growth in an immune animal, a white rat. He also changed a slightly virulent pseudodiphtheria type to a typical diphtheria form with increased virulence by one passage through a guinea-pig. Denny (1903) made a series of frequent microscopic examinations during the early growth of B. diphtheriae, B. pseudodiphtheriae, and B. xerosis. He found that in young cultures all three present striking similarities showing a large proportion of D^2C^2 types. In older cultures, the true diphtheria and xerosis bacilli displayed developmental differences characteristic of the higher bacteria, such as granulation, segmentation, etc., altho the forms of the xerosis bacilli were usually shorter and thicker than those of the Klebs-Löffler bacilli. Hofmann's bacillus always remained typical in form. He found that unfavorable conditions, such as symbiosis with a large number of other organisms, delayed the formation of granular types and suggests that this may explain the greater abundance of solid forms in the 15-hour cultures as convalescence advances and the proportion of ordinary throat bacteria increases.

Graham-Smith (1903) reports that some B. hofmanni are clubbed and often slightly curved but are broader and take the stain more deeply than B. diphtheriae. They sometimes show stained segments which are very dark and well defined, the septa being narrow and running in all cases transversely across the bacilli. These forms always revert to the typical D^2 type. Smirnow (1908), in using double-walled celloidin sacs to study symbiotic relations of B. diphtheriae with other organisms, recovered coccoid forms from the compartment originally containing the diphtheria bacilli. These reverted to typical form, however, after growth in broth and then on blood-serum.

Pathogenicity.—Löffler considered this the most unvarying feature of the diphtheria organism. The gelatinous edema in the subcutaneous tissue at the point of inoculation, the congestion of various organs, and more especially the hyperemia and enlargement of the suprarenal capsules are certainly highly characteristic. Roux and Yersin (1890) were able to increase the virulence of slightly virulent diphtheria bacilli by injection together with erysipelas streptococci. They were unable to do this with non-virulent organisms, however. Trumpp (1896) transformed an avirulent D^2 type to a typical diphtheria organism by passage through three guinea-pigs together with a non-fatal dose of diphtheria toxin. His organism produced acid, however, and he does not believe it possible thus to transform a non-acid former.

Hewlett and Knight (1897) changed a typical acid-producing virulent diphtheria culture into a non-virulent D^2 by heating for seventeen hours at 45° C. By prolonged cultivation for 20 generations and then long incubation and passage through a guinea-pig they also changed a typical non-acid-producing D^2 into an acid-producing typical diphtheria organism. They were unable to repeat these experiments successfully.

Bergey (1898) was unable to give virulence to D^2 by passage through several animals. Martin (1898) increased the virulence of slightly virulent diphtheria organisms by growth in celloidin sacs in the body cavity of rabbits. Salter (1899) by five successive passages through goldfinches exalted the virulence of four separate strains of typical pseudodiphtheria bacilli to a point where they were capable of killing guinea-

pigs. The transformation was complete both as to morphology and acid production. The toxic action was entirely neutralized by diphtheria antitoxin. Nineteen strains of pseudodiphtheria bacillus proved virulent for goldfinches and chaffinches, 18 out of the 19 were virulent for canaries, and many of the strains were virulent for other birds.

Davis (1898) isolated 12 strains of virulent pseudodiphtheria organisms which produced a general bacteremia and against which diphtheria antitoxin afforded no protection. Wesbrook, Wilson, and McDaniel (1900) report an outbreak of diphtheria in which the type most frequently found resembled D². They all produced acid, however, in dextrose broth and the toxin formed was neutralized by diphtheria antitoxin. Gorham (1901) says that type D² has sometimes proved pathogenic to guinea-pigs. Cobbett (1901) isolated and tested 86 strains of the pseudo-diphtheria bacillus. They all proved to be non-virulent in doses of 2 c.c. of a 48-hour culture. Lesieur (1901), as already noted above under "Morphology," was able to make several strains of pseudodiphtheria bacilli virulent and also accomplished the reverse transformation. Neumann (1902) examined 78 strains of D² all of which proved to be non-virulent. Ohlmacher (1902) transformed a slightly virulent D² type by one passage through a guinea-pig and at the same time increased its virulence.

Williams (1902) made a careful series of investigations extending over a period of about seven years. Inoculations of large doses of typical D² proved to be innocuous to goldfinches. Successive peritoneal inoculations of D² produced no exaltation in virulence. Four strains of typical diphtheria bacilli showed no change after growing in an immune host (white rat) for 48 hours. Two non-virulent but morphologically typical strains of diphtheria organisms were grown with virulent streptoccoci in broth for 90 generations, transplanted every three or four days. When separated, no change whatsoever was observed. Two cultures of Klebs-Löffler bacilli were cultivated for several months at 40° C., transplanting them every week, and six cultures were grown for the same length of time at 43° C.—45° C., alternating every week to a temperature of 35° C. These organisms were somewhat smaller at the high temperatures but reverted to their normal size when placed at body heat. The author observed no sequence of types during the course of the disease and considered the morphology of the several species to be quite distinct.

Graham-Smith (1904) observed no partially attenuated diphtheria bacilli, altho 25 strains out of 113 which he examined were entirely non-virulent. Ruediger (1903) and Hamilton (1904) describe three varieties of pseudodiphtheria bacilli, one of which is pathogenic to guinea-pigs. This produces a general bacteremia and is neutralized by the serum of a rabbit immunized against any of the virulent varieties, but not by regular diphtheria antitoxin. Wesbrook (1905) himself states that in dealing with epidemics, bearers of all forms other than A, C, and D may be safely disregarded. Hadley (1907) recovered D² forms from inoculations of three strains of virulent C, C¹, and C² forms. Also one of his D² types proved to be virulent but, when recovered after inoculation, proved to be of the barred type. Corbett (1906) states that D², E², and C² types generally prove to be virulent. Zinsser (1907) found no virulent pseudodiphtheria bacilli.

No exhaustive analysis of this literature is necessary to make clear the fact of the many discrepancies in the results obtained by different observers. The fermentative action of the true diphtheria bacillus

upon dextrose and some other carbohydrates and the characteristic toxic action, however, stand out as the features about which there is the least dispute. It is almost universally conceded that B. hofmanni produces little or no acidity in dextrose broth and is usually non-pathogenic to guinea-pigs or at most produces only a slight local edema at the point of inoculation. With the true Klebs-Löffler bacillus, however, the toxic action is peculiarly characteristic and it generally produces acid in dextrose broth.

In view of the contradictory observations and diverse opinions in regard to the relation of the members of this group, the writer determined to investigate further the question as to the identity of the pseudodiphtheria and the diphtheria bacillus. The investigation was not extended to a consideration of B. xerosis and the other diphtheroid bacilli.

STATEMENT OF THE PROBLEM TO BE SOLVED.

The crux of the situation then seems to be: Can a typical non-acid-producing non-pathogenic Hofmann's bacillus be changed into a typical acid-producing pathogenic Klebs-Löffler bacillus whose toxic action can be neutralized by commercial diphtheria antitoxin? And if this is true in the laboratory, does it necessarily hold true in nature? Further, does the reverse transformation take place? Do the varieties of virulent diphtheria change through the course of the disease from the granular to the segmented type, and, becoming less and less virulent, finally take on the form of the non-virulent pseudodiphtheria bacillus?

EVIDENCE BEARING ON THE RELATION OF THE PSEUDODIPHTHERIA AND THE DIPHTHERIA BACILLUS.

From a series of cultures taken during the course of the disease.—A tabulation of the Wesbrook types obtained from smear preparations of cultures was made. These cultures were taken mostly from persons suffering from the disease, altho a few were from contacts. Most of the examinations were made by the writer as part of the routine work in the laboratory of the Rhode Island State Board of Health. Some, principally those taken toward the end of the disease from cases in Providence, were made by Professor F. P. Gorham, bacteriologist of that city. The results are shown in Table 1.

These results, while not sufficient to draw any far-reaching conclusions, seem to point to a decision against the Wesbrook-Gorham theory. Solid-staining C^2 and D^2 forms are found fully as often in the earlier cultures as in those taken when the patient was convalescing. Another point to be noticed is that the same types were not constantly present but appeared to vary considerably from time to time.

In this connection, it is worthy of note that during the last year or two, the organisms from clinical diphtheria cases in Providence have very frequently been barred forms instead of granular types as was formerly the case. If, as some advise, a positive diagnosis should be given only when the granular forms are present, a large percentage of the cases of clinical diphtheria in this vicinity would be overlooked.

From attempts to establish virulence in a non-virulent organism.—The first step was to obtain a number of pure cultures of B. hofmanni. These were isolated, some from cultures sent in to the Rhode Island State Board of Health Laboratory for diagnosis but mostly from cultures from contacts obtained from the City of Providence Laboratory. Following is a list (Table 2) of the different strains isolated with their action on guinea-pigs and reaction to phenolphthalein when grown in one per cent dextrose broth for three days.

Most of these cultures were tested for purity several times before using them. During the first part of this investigation the cultures for inoculation were grown in ordinary nutrient broth. Subsequently, however, they were always grown in dextrose-free broth in order to have the media as favorable as possible for toxin formation. A growth of 48 hours was used in most cases, for inoculation, but occasionally a longer growth was employed, especially when the two-day cultures were not well grown. Smear preparations were in all cases stained with Löffler's methylene blue.

The 23 cultures of typical B. hofmanni (Nos. 1–9 inclusive and 14–27 inclusive) were tested for virulence upon half-grown guinea-pigs. They all proved non-virulent or only slightly virulent (i.e., producing local edema only) in doses of one per cent of the body weight. Most of the strains produced a slight local edema at the point of inoculation and some of the guinea-pigs showed a small loss

TABLE 1.

TABULATION OF SUCCESSIVE CULTURES.

CLINICAL CASES.

Name	First Culture	No. of Days Intervening	Second Culture	No. of Days Intervening	Third Culture	No. of Days Intervening	Fourth Culture	No. of Days Intervening	Fifth Culture	No. of Days Intervening	Sixth Culture
E. S. C.	C^1D^2	1	D^2C^2	5	$D^2C^2A^2$						
W. B.	$A^1C^1C^2$	11	$C^1C^2A^2D^2$								
C. B.	C^2D^2	13	$CC^1C^2D^2$								
E. D.	$C^1C^2D^2$	5	C^2D^2	3	$C^1C^2D^2$						
E. T.	$C^1C^2D^2$	11	D^2	5	D^2						
L. T.	C^1C^2	11	C^2								
L. D.	$C^1C^2D^2$	7	D^2								
H. W.	C^1C^2	5	C^1A^2(few)D^2								
M. D.	C^1C^2	19	CC^2	6	C^2						
P. R. L.	C^2D^2	15	C^1C^2	3	C^1C^2	4	$C^1C^2D^2$				
R. L.	C^1	9	$C^1A^1C^2$								
L. C.	$C^1C^2A^1$	14	$C^1C^2A^1$	8	C^1C^2						
S. C.	$C^1C^2D^2$	5	C^1C^2	3	C^2	1	C^2	6	C^2	2	C^1A^1
								2	$C^1C^2A^1A^2$	2	C^2
R. M.	C^1C^2	16	C^1C^2	6	C^1C^2						
Mrs. B.	$C^1C^2D^2$	16	C^2	1	C^2						
A. C.	C^1	13	C^1C^2	3	C^2D^2						
J. B.	C^1C^2	19	C^1C^2	4	C^2						
M. P.	$C^2D^2C^1$	13	$C^2D^2C^1$								
M. C.	C^2D^2	2	C^1A^1	3	D^2						
J. H.	C^1C^2	4	C^1A^1	4	C^2	3	$C^1C^2D^2A^1$				
J. V. G.	C^2C^1	12	D^2								
F. W.	C^1	20	D^2	5	D^2C^2						
C. M.	$D^2C^2C^1$	3	D^2C^1								
A. B.	CD	6	C^1C^2	3	D^2C^1	2	D^2	2	D^2C^2		

CONTACT CASES.											
E. H. W.	D^2 (nose)	2	D^2 (throat)	2	D^2 (nose)	5	D^2 (throat)		D^2 (throat)	2	E^2 (nose)
			D^2C^2 (nose)				D^2 (nose)	3	C^1A^1 (nose)		
W. T. W.	D^2 (nose)	2	D^2C^2 (nose)	4	D^2 (throat)		D^2 (throat)		D^2C^2 (throat)		$D^2C^2C^1$ (throat)
					D^2 (nose)	6	D^2C^2 (nose)	2	D^2 (nose)	5	$D^2C^2C^1$ (nose)
										1	D^2 (throat)
E. V. E.	C^2	2	C^1C^2	3	C^2	1	C^2A^2	7	D^2		
CLINICAL CASES.*											
M. P.	C^1D^2C	3	D^2C^1A	5	D^2C^1A	5	D^2C^1				
F. P.	D^2C^1	3	C^1D	5	D^2C^1	5	D^2				
P. C.	CDC^1	4	D^2								
O. C.	D^2C^1	2	D^2C^1	2	D^2C^1	2	D^2C^1A				
CONTACT CASES.											
S. K.	D^2C^1	5	D^2DC^1	1	$C^1C^2D^2$	1	D^2C^1	6	D^2C^1D	4	D^2
Mr. P.	C^1C^2	1	CC^1C^2	1	C^1C^2D						
J. K.	D^2C^1D	2	D^2C^1	3	C^1C^2						
B. K.	D^2C^1	2	D^2C^1	3	D^2C^1	2	C^1D^2	2	D^2C^1	1	D^2C^1
W. D.	D^2C^1C	2	CC^1	3	C^1	2	D^2CC^1				
M. O.	CC^1D	7	C^1D^2								
A. D.	D^2C^1	2	D^2C^1	3	D^2						
Miss P.	D^2C^1	1	D^2C^1C	3	C^1C^2	1	C^1C^2				
A. C.	CC^1	2	D^2C^1	3	C^1CD						
J. K.	C^1D^2	1	C^1D^2	1	C^1D^2						

* Examinations hereafter were made by Professor F. P. Gorham.

of weight. These signs were very transitory and it was found to be difficult to recover the organism if allowed to remain within the body for more than 18 to 24 hours. All but one of these strains produced

TABLE 2.

No.	Source	Organism	Virulence	Isolated from:	Reaction in Dextrose
1	E. P.	B. hofmanni	Non-virulent	Diphtheria	Not tested
2	X.	"	"	"	" "
3	S. H.	"	"	Contact	0
4	M. W.	"	"	"	0.05
5	C. G.	"	"	S. F. Contact	0.15
6	J. DeZ.	"	"	" "	Not tested
7	I. M.	"	"	Diphtheria	" "
8	G. P.	"	"	Contact	0.15
9	A. S.	"	"	"	Not tested
10	E.	B. diphtheriae	"	"	1.05
11	McD.	"	Virulent	Diphtheria	0.50
12	B.	"	"	"	0.85
13	J. L.	"	"	"	0.53
14	S. S.	B. hofmanni	Non-virulent	Contact	0.05
15	E. S.	"	"	"	0.15
16	W. G.	"	"	"	0.20
17	M. T.	"	"	"	0.10
18	L. M.	"	"	"	0.05
19	E. B.	"	"	"	0.15
20	R. B.	"	"	"	0.15
21	E. U.	"	"	"	0.13
22	A. B.	"	"	"	0.20
23	P. V.	"	"	"	0.05
24	D. V.	"	"	"	0.15
25	E. C.	"	"	"	0.20
26	F. U.	"	"	"	0.10
27	T. P.	"	"	"	0.05
28	K. W.	B. diphtheriae	Virulent	Diphtheria	1.20
29	V. McA.	"	"	"	1.50
30	C. M.	"	"	"	1.30
31	XXXI	"	"	"	0.90
32	27y	"	"	"	Not tested

some acidity in dextrose broth with phenolphthalein as an indicator, but the amount was so slight in each case that the reaction would have been alkaline to litmus, the indicator used by most observers.

1. BY SUCCESSIVE PASSAGES THROUGH SUSCEPTIBLE ANIMALS.

Guinea-pigs.—Serial inoculations were tried with a number of strains of B. hofmanni. They were allowed to stay in the body of one guinea-pig as long as possible and still be recovered. Twenty-four hours, as mentioned above, was about the average length of time. The organism recovered was then grown in broth and again inoculated. Strain 1 was in this way passed through four guinea-pigs; No. 5, through ten; No. 6, through eight; and No. 8, through six. The result in all cases was the same. Occasional barred forms

were observed but no permanent change was produced, the barred forms appearing in the culture recovered from one animal and not appearing in the succeeding culture at the time of inoculation or in the next culture recovered. The organisms were the same morphologically and were as non-virulent to guinea-pigs at the end as at the beginning of the series.

Canaries.—In carrying on inoculation experiments with canaries, two-fifths to one-half of a c.c. of a 48-hour culture was the amount generally used. The birds were Harz Mountain canaries weighing about 15 gm. each, the dose, therefore, being about three per cent of their body weight. Nine strains of B. hofmanni (Nos. 4, 5, 9, 15, 16, 18, 20, 22, and 25) were inoculated into as many canaries intramuscularly, all proving to be non-virulent. Some subcutaneous inoculations were tried with no very great success because of the extremely small amount of subcutaneous tissue and thin integument. When so inoculated, however, a very slight edema was produced. Several successive inoculations were made with No. 9 but it was found to be very difficult to recover the organism even if the canaries were killed in 18 hours. One inoculation of 1 c.c. (No. 16) was made, putting $\frac{1}{2}$ c.c. into each pectoral muscle. Even this large amount, about seven per cent of the body weight, produced no ill effect, while $\frac{1}{2}$ of a c.c. of a virulent diphtheria culture (Strain 13) killed a canary in 24 hours. In all, 15 canaries were inoculated with the various strains of B. hofmanni mentioned. Quite contrary to Salter's results, not one strain proved virulent to these birds.

Pigeons.—Five strains of the pseudodiphtheria bacillus (Nos. 1, 5, 6, 8, and 9) were inoculated into pigeons intramuscularly. Little if any edema was produced and it was found necessary to kill the pigeon in about 15 hours after inoculation in order to recover the organism. A short series of successive inoculations was made with strain No. 1, but it was evident that pigeons were even less susceptible than guinea-pigs to the action of B. hofmanni. Three strains of virulent diphtheria organisms (11, 12, and 13) were inoculated into as many pigeons. The one inoculated with No. 13 died in two days with marked whitening and degeneration of the muscle fibers. The other two pigeons were apparently not much affected by the dose which was over one per cent of their weight. As these two strains

had killed guinea-pigs in two and three days respectively, it would seem that pigeons may be somewhat immune to diphtheria toxin.

Chickens.—Three strains of B. hofmanni (Nos. 5, 8, and 9) were inoculated intramuscularly. No ill effects were observed. In several subsequent inoculations it was found impossible to recover the organism even in 20 hours. Great care was taken, portions of the muscle being removed aseptically and incubated on blood-serum, but the organism had evidently disappeared. Virulent diphtheria organisms (Strain 11) were three times inoculated into chickens. The first died in sixteen days, the second in three days, and the third in four days. In all cases the muscle showed very marked degeneration, the tissues being yellowish white and friable, but even with great care only in the last case were the organisms recovered. Chickens are apparently not at all susceptible to the pseudodiphtheria bacillus and while they are evidently susceptible to the diphtheria toxin, the difficulty with which the organisms were recovered would seem to throw doubt upon the possibility of the transmission of the disease between fowls and humans.

2. BY THE INOCULATION OF LARGE DOSES AND BY THE USE OF IMMATURE ANIMALS.

Large amounts of broth cultures were next inoculated. No. 9 was passed successively through three guinea-pigs using $3\frac{1}{2}$ per cent, 4 per cent, and 7 per cent of the body weight. Two guinea-pigs were inoculated with 2 per cent and 4 per cent respectively of No. 8. As diphtheria is so largely a disease of children, it was thought that very young guinea-pigs might be more susceptible. Accordingly several very small guinea-pigs were inoculated with doses as high as 7 per cent of their body weight. A large amount of edema was caused by all of these excessive inoculations. This generally extended over a large portion of the ventral surface of the body but apparently no effect other than this local disturbance was produced. The type of the organisms remained the same and the edema had disappeared by the end of 24 to 48 hours.

3. BY SENSITIZING CULTURES WITH HOMOLOGOUS SERUM.

According to White and Graham (1909), the infective power of the tubercle bacillus for rabbits was increased by sensitization of the organisms with normal rabbit serum. Their method was given a

brief trial on the pseudodiphtheria bacillus. Blood was collected from guinea-pigs aseptically and the serum pipetted off. One blood-serum culture of Strain 16 was emulsified in 5 c.c. of this serum, incubated for 30 minutes, and inoculated into a guinea-pig. The same procedure was followed with Strain 22 with an incubation of 45 minutes. Neither inoculation had apparently any more effect on the guinea-pigs than when the organisms were not so treated.

4. BY GROWING CULTURES IN AN INCREASED SUPPLY OF OXYGEN.

It is well known that B. diphtheriae produces toxin more plentifully when there is a large supply of air. Presumably it is the oxygen of the air which is advantageous. It was thought that possibly an increased amount of oxygen might encourage the production of toxin by the pseudodiphtheria bacillus. This was accordingly tried.

Inoculated broth cultures were placed in a vacuum desiccator from which the air was exhausted by means of a water pump, until a pressure of about 12 m.m. was obtained. This was measured by a manometer and then a measured amount of oxygen was permitted to pass in. Three strains of B. hofmanni were incubated for three days with 40 per cent of oxygen, and three others with about 80 per cent. Guinea-pigs inoculated with the cultures so treated showed no unusual effects.

5. BY GROWING CULTURES IN CELLOIDIN SACS IN A SUCCESSION OF ANIMALS.

It was next determined to try a series of experiments using the well-known celloidin sac method, with the hope that a longer growth in the body of a susceptible host might tend to increase virulence. These sacs were made according to the usual technic with a few minor exceptions. It was found to be better not to fill the perforated glass tube with water in order to force off the capsule by the breath (Gorsline, 1903), but to draw it off by careful pressure and manipulation with the fingers, using the perforation at the end of the tube merely as a means for the ingress of air to prevent the collapse of the sac. The sacs were not placed in water at all until they had been constricted on to the end of the glass tube and were ready for sterilization. During the first experiments with this method, the capsules were incubated in sterile broth both before and after removal from the

peritoneal cavity. In all cases, no growth was observed in the broth thus controlling the accuracy of the technic.

Strain 5 was passed through three successive guinea-pigs by this method. The first capsule remained in the peritoneal cavity 11 days. It was then removed, the organism recovered and placed in a new capsule which remained in a second pig for 14 days. This process was repeated in a third pig for 7 days, making a total of 32 days that this strain grew in the body of a susceptible animal. No effects were observed either on the pigs or on the organism. A few segmented forms were recovered from the second capsule but were not present in the culture recovered from the last.

It was thought that the insertion of a sac filled with the pseudodiphtheria bacilli together with a simultaneous peritoneal inoculation of virulent diphtheria organisms might produce the proper body fluids for the desired metamorphic change. In this way, the pseudodiphtheria culture would not be contaminated, nor be destroyed by the phagocytes. Both the diphtheria toxin and the body fluids of an animal suffering from the disease must necessarily come into contact with organisms so treated.

Strain 5, a pseudodiphtheria bacillus, and Strain 11, a virulent diphtheria bacillus, were accordingly inoculated in this manner. The pig died in two days with the characteristic symptoms of diphtheria poisoning but only D^2 forms were recovered from the capsule. It was attempted to prolong the time in which the pseudodiphtheria bacillus should remain bathed in these fluids by the insertions of two capsules, one containing B. diphtheriae and the other the pseudodiphtheria bacillus. Strains 5 and 11 were again used. The capsules were small, containing not much over $\frac{1}{2}$ c. c. each. Altho the pig lost about 100 gm. in weight during the week following, it did not die. It was killed at the end of the week and organisms as typical as when they were inserted were recovered from the respective capsules. These recoveries were again placed in capsules and grown in the peritoneal cavity of another pig for seven days. The result was the same as before. Similar experiments were performed with Strains 3 and 11, pseudodiphtheria and true diphtheria cultures, respectively, passing the organism through two pigs, nine days in the first and eight in the second. Strain 5 was also tried again,

using Auroccocus aureus in the other capsule to see what effect the symbiosis with that organism would have. The results of all the experiments were the same. The pseudodiphtheria bacillus still retained its usual characteristics.

6. BY INOCULATION OF ANIMALS IN COMBINATION WITH TOXIN, DIRECTLY OR IN CELLOIDIN SACS.

Believing that the presence of toxin might induce the pseudodiphtheria bacillus to become virulent, a celloidin capsule containing a broth culture of Strain 16 was inserted into the body cavity of a guinea-pig together with a peritoneal inoculation of 1 c.c. of sterile weak diphtheria toxin. This toxin had been obtained by the ordinary process of filtering a culture through a Pasteur filter. It had been proved sterile by incubation and by inoculation on blood-serum. Eighteen days later this pig was inoculated subcutaneously with $\frac{1}{4}$ c.c. of a more potent sterile diphtheria toxin, the minimum lethal dose of which was about 0.2 c.c. The pig died the next day with characteristic symptoms of diphtheria poisoning, and typical pseudodiphtheria bacilli with an occasional barred type were recovered from the capsule.

The same strain (16) was placed in a capsule in the peritoneal cavity of a guinea-pig together with a capsule containing the more potent toxin. The pig died in two days. D^2 was recovered.

Three capsules were placed in the body cavity of one pig, one capsule containing Strain 20; the second, Strain 16; and the third, toxin. The pig was killed and autopsied at the end of the fourth day. The adrenals appeared characteristic for diphtheria poisoning but typical B. hofmanni were recovered from the two capsules in which they had been placed.

A four-day culture of Strain 4 was inoculated together with $\frac{1}{4}$ c.c. of the toxin. The guinea-pig died in about 30 hours, the recovery showing typical D^2.

Strain 3 was inoculated together with $\frac{2}{5}$ c.c. of this toxin. The guinea-pig inoculated died in 24 hours. Two blood-serum cultures recovered from this pig were emulsified in dextrose-free broth and this was again inoculated together with $\frac{1}{5}$ c.c. of the toxin. Upon the death of the guinea-pig in 40 hours, the process was repeated

with a third pig. The organisms recovered from all these inoculations were typical B. hofmanni.

7. BY A PROCESS OF SELECTION OF THOSE TYPES APPROACHING THE MORPHOLOGY OF VIRULENT ORGANISM FOR A SERIES OF "GENERATIONS."

Two strains of pseudodiphtheria bacilli were chosen, one, Strain 15, because it generally showed small organisms with blunt ends, and the other, Strain 16, because it usually displayed long forms with more or less pointed ends and a larger number of barred types than any other strain among those tested by the writer. In other words, Strain 15 looked the least like the true diphtheria bacillus and Strain 16 the most like that form.

These strains were streaked out on blood-serum, incubated, and the following day from 20 to 30 individual colonies were fished and inoculated on blood-serum tubes. After growth for slightly varying periods but usually about 16 to 20 hours, the cultures were examined microscopically and that one which possessed the largest types and segmented forms, if any were present, was selected and the process repeated. This was carried out 12 successive times with the following results.

Strain 15 occasionally showed longer forms but there was no continuous sequence. Strain 16 showed a few segmented forms in a large number of cultures throughout the series. They seemed to be more plentiful when the cultures happened to be incubated for a longer period than usual and also when there were only a few comparatively large colonies in a tube. These two conditions are similar, for in one we have the individual organism producing a large number of generations because of long-continued incubation, and in the other we have the same result because of more favorable conditions of growth.

The cultures from the seventh selection of Strain 16 were incubated two days and on examination one tube in particular, which had only a few large colonies, showed an unusual number of types resembling C^1 and A^1. The next lot of cultures from this selection were incubated about 20 hours and only one or two segmented types were observed in any of the smears. These cultures were replaced in the incubator and the growth continued up to 65 hours. A marked

change of types had occurred in all of the tubes. Some of the tubes showed in the smears from 20 per cent to 40 per cent of barred types, many of them taking the stain rather poorly (Fig. 1).[1] The next series of cultures from the selection of this date, however, was incubated the usual length of time, and on careful examination, scarcely

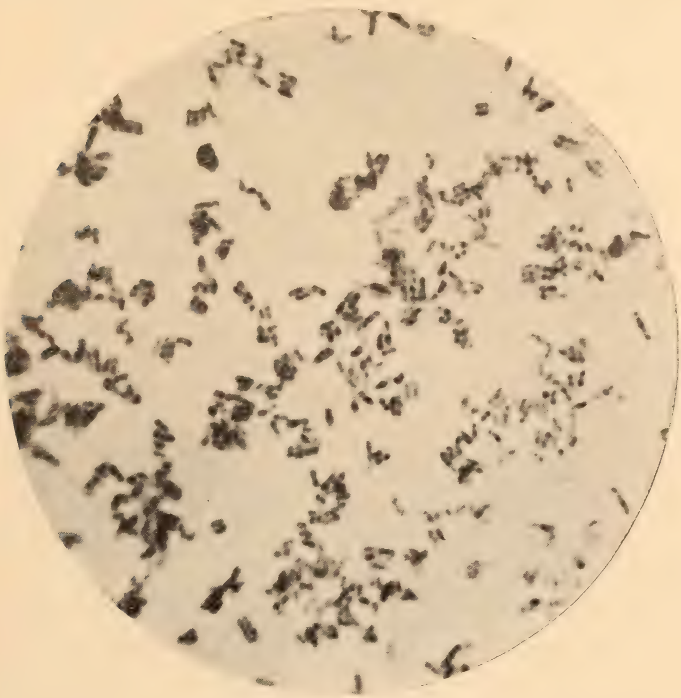

FIG. 1.—Strain No. 16. B. hofmanni. Showing barred and faintly staining involution types in a culture from the eighth selection grown 65 hours. ×2,000.

a barred form could be found. Strain 15, which was put through the same procedure, showed no such change of types during the long incubation but retained its usual appearance. At the end of the 12 selections, both strains presented the same morphology as originally and were still non-pathogenic to guinea-pigs.

The results of this series together with a number of other minor observations led the writer to believe that possibly B. hofmanni might at times pass through a cycle of morphological changes similar

[1] I am indebted to Dr. Edward Leaming for the photomicrographs.

to those described by Denny (1903) for B. xerosis and B. diphtheriae. Accordingly it was decided to repeat a portion of his work.

From a study of the types of the organism appearing at different stages in the growth of cultures.—Four strains of B. pseudodiphtheriae (Nos. 3, 4, 16, and 19), three strains of virulent B.

FIG. 2.—Strain No. 12. Virulent B. diphtheriae. Eight hours' growth. ×2 000.

diphtheriae (Nos. 11, 12, and 13), and one strain (No. 10) of non-virulent B. diphtheriae were used. Denny's technic was followed carefully. Emulsions in broth were made from 24-hour cultures of these organisms and several loopfuls of this mixture smeared over the surface of moist blood-serum. The cultures were incubated at body temperature and smears made from each at the end of 4, 8, 12, 15, 27, 49, and 72 hours. Different parts of the surface of the serum were used for each preparation and the morphology of the organisms carefully noted.

The results differed from those obtained by Denny only in minor details.

Four-hour cultures.—No growth was visible but a number of bacilli were found in the microscopical preparations. These were more abundant in the diphtheria than in the pseudodiphtheria cultures. The organisms at this state were all solid-staining. The pseudodiphtheria bacilli were all typical D^2. In the diphtheria cultures, also, D^2 predominated with one or two C^2 forms in Strain 10 and many short thick, solid-

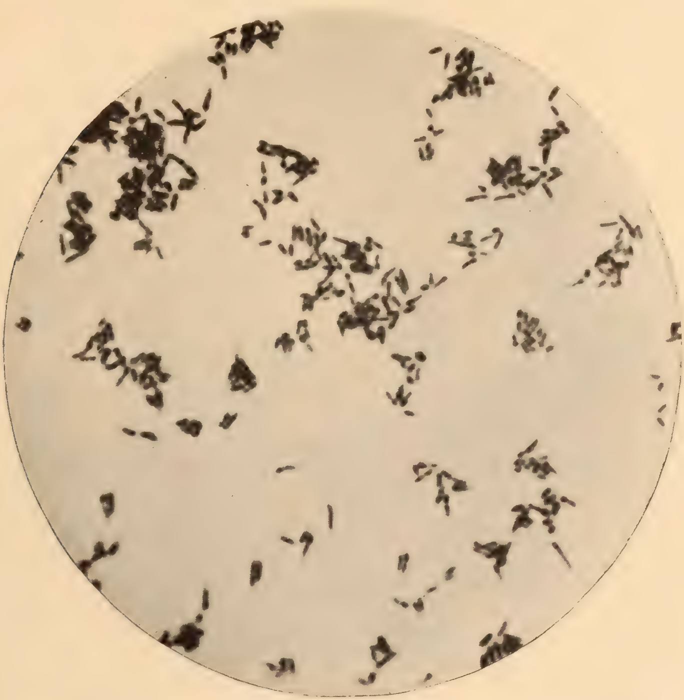

FIG. 3.—Strain No. 12. Virulent B. diphtheriae. Twelve hours' growth. ×2,000.

staining clubs (A^2) particularly in Strain 12. The D^2 forms from the true diphtheria cultures were apparently a little more curved and pointed at the ends than those in the pseudodiphtheria preparations at this stage.

Eight-hour cultures.—The growth was barely visible on some of the tubes. The bacilli were all solid-staining, D^2 being the only type found in the pseudodiphtheria cultures. In the diphtheria cultures (see Fig. 2) the bacilli were longer, making a larger proportion of C^2 forms. Many of the D^2 forms were curved, some of them being thick and swollen either at the ends or in the middle. Fewer of the short A^2 types are present than in the four-hour stage.

Twelve-hour cultures.—The growth was distinctly visible. The bacilli from the pseudodiphtheria cultures were all of the D^2 type. Those from the diphtheria cultures

(see Fig. 3) were longer with a number of C^1 types. There was still a large percentage of D^2 types, however.

Fifteen-hour cultures.—The bacilli from the pseudodiphtheria cultures were typical D^2. The diphtheria organisms (see Fig. 4) exhibited some granular types along with the C^1, C^2, D^2, etc.; D^2 was still present in considerable numbers, particularly in Strain 13.

Twenty-seven-hour cultures.—In this state of development, the pseudodiphtheria bacilli, with the exception of Strain 16, were noticeably shorter than in the earlier stages.

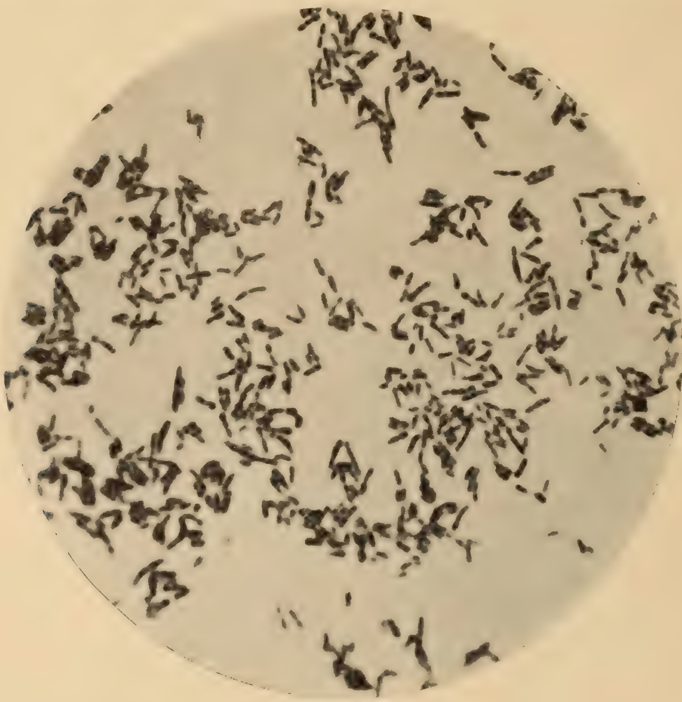

FIG. 4.—Strain No. 12. Virulent B. diphtheriae. Fifteen hours' growth. ×2,000.

The diphtheria cultures (see Fig. 5) contained more granular types. There was still a predominance of barred types, Strain 13 showing no granular forms whatsoever. D^2 was still present in three of the cultures, the only one in which it was not present being Strain 10, the non-virulent strain.

Forty-nine-hour cultures.—All but Strain 16 of the pseudodiphtheria strains displayed the short forms. In that culture the length of the earlier growths was retained and a few C^1 forms were observed. The B. diphtheriae showed no marked difference in morphology except that there were more granular forms. Many of the bacilli of both species took the stain poorly.

Seventy-two-hour cultures.—No particular differences were observed except that more organisms were faintly stained, more C^1 forms were observed in Strain 16, and a very few in Strain 21.

The results obtained coincided very exactly with Denny's except that the cultures as a whole seemed to develop a little more slowly than his. The solid-staining forms of the true diphtheria bacillus, comprising all of the organisms grown in eight-hour cultures and a considerable number in the cultures of longer incubation of some strains, would certainly be confused with the similar types of the

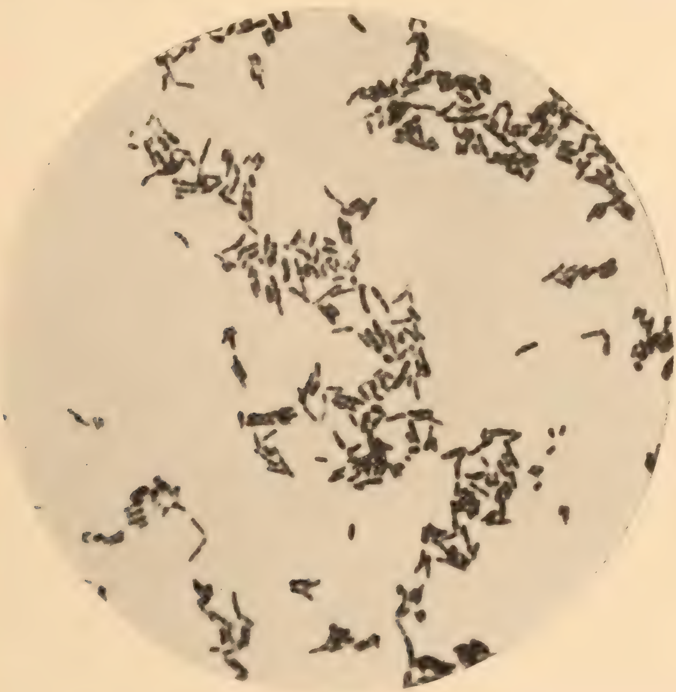

FIG. 5.—Strain No. 12. Virulent B. diphtheriae. Twenty-seven hours' growth. ×2,000.

pseudodiphtheria bacillus when found in throat or nose cultures. In these strains of the diphtheria bacilli, the D^2 types seemed to persist longer and there were more C^1 forms than in those observed by Denny. Also, it was found that a few C^1 forms were present in the older cultures of two strains of the pseudodiphtheria bacilli.

In this connection, the laboratory history of Strains 31 and 32 are of interest. Strain 31 was isolated from a clinical diphtheria case and after incubation for about 16 hours showed a morphology so closely resembling that of B. hofmanni that it was so regarded.

This culture remained standing at room temperature for about two weeks. Upon re-examination, it still showed D^2 forms. It was grown in broth for 48 hours and inoculated into a rabbit, killing the animal in four days. Both the blood-serum culture from the broth at the time of inoculation and the culture recovered from the rabbit showed typical diphtheria organisms. Subcultures and pure cultures

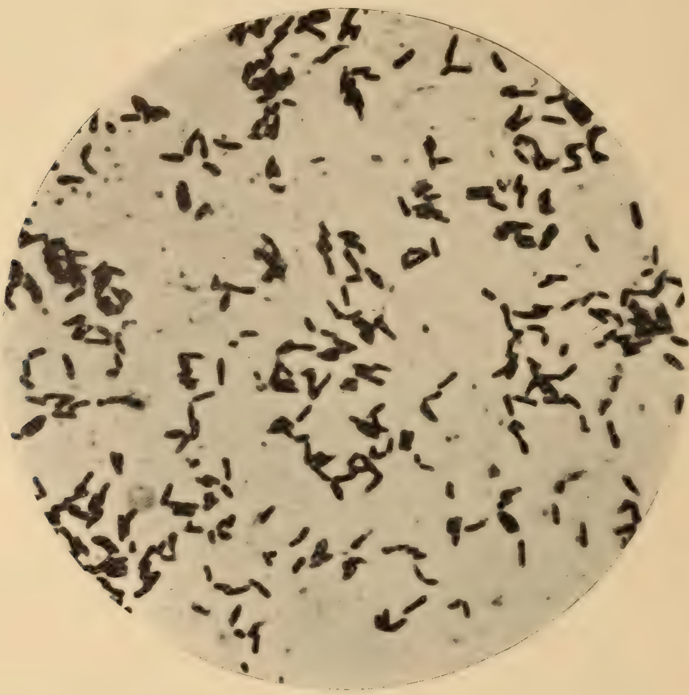

FIG. 6.—Strain 31. Virulent B. diphtheriae. Solid staining D^2 types in culture incubated for sixteen hours. ×2,000.

from the original culture, in all except one instance, showed typical diphtheria organisms. That one showed long D^2 forms after incubation for about 16 hours (see Fig. 6). On replacing this culture in the incubator, however, and growing for another 24 hours, the morphology became typical for B. diphtheriae.

Strain 32 was isolated from a case of clinical diphtheria displaying large A, A^1, and C types. It was inoculated into a guinea-pig; the pig died; and the organisms recovered were typical D^2 types. The

culture recovered from this guinea-pig was again inoculated into a guinea-pig, killing the pig in 35 hours with symptoms characteristic for diphtheria poisoning. $D,^2$ C^2 were the types recovered (see Fig. 7).

Still another item, in connection with morphological changes, may be worthy of comment. Two strains of B. hofmanni (8 and 9) were grown in large, tall blake bottles containing slants of Löffler's blood-

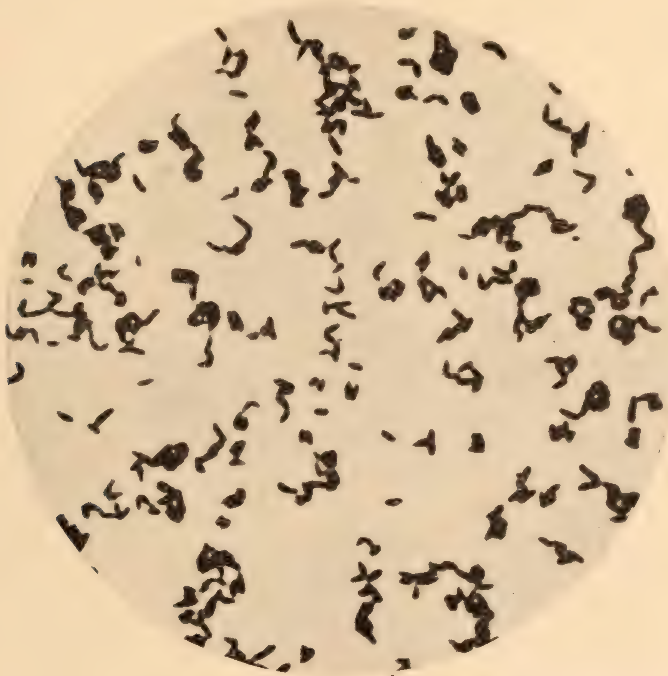

FIG. 7.—Strain No. 32. Virulent B. diphtheiiae. Solid staining types recovered from guinea-pig which showed characteristic symtoms of diphtheria poisoning. ×2,000.

serum, for one week at 37° C., and for five weeks at room temperature. Examinations at the end of that period showed large club and dumb-bell shaped forms with bars and granules much resembling similar types of B. diphtheriae. A 48-hour broth culture from the bottle culture of Strain 9 was inoculated into a guinea-pig. As the animal showed no symptoms of illness, it was killed the next day and typical B. hofmanni were recovered. Strain 8 immediately reverted to its original form on being inoculated on a fresh blood-serum tube.

From a study of the frequency curves of acid production of the pseudodiphtheria and diphtheria organisms.—The biometric method of studying variations having been so successfully used by Winslow (1908) in determining the systematic relationships among the Coccaceae, it was thought that that method might throw some light upon the conditions in this group. The action of the organisms on dextrose broth was chosen as being a character that was definitely measurable by titration and, therefore, best adapted for work of this sort.

One hundred and thirty-one pure cultures of the pseudodiphtheria bacillus obtained in carrying out the selection experiments described above, and 35 subcultures from the other strains tested in this investigation, were used in constructing the curve of B. pseudodiphtheriae. Sixty-two cultures of B. diphtheriae, mostly pure cultures from Strains 12, 13, and 28 and a few from the other strains, were used to determine the curve for that organism.

The cultures were all grown in 1 per cent dextrose broth and incubated at 37° C. for 72 hours. They were then boiled one minute to drive off the carbon dioxide and immediately titrated with N/20 NaOH, phenolphthalein being used as the indicator. In all cases a considerable number of blanks were run and the average subtracted from the titration figures to obtain the amount of acid actually produced by the organisms. The results of this experiment are given in Tables 3 and 4, arranged in ascending scales according to the amount of acid produced. Considerable variation will be noticed in the amount of acid produced by B. hofmanni but in no case is it in sufficient quantity to be acid to litmus, the indicator which has been used by most observers. Still wider variation is displayed by B. diphtheriae, many cultures not producing enough acid to turn litmus solution. This may be partly due to poor growth, altho Goodman (1908) obtained many results as low as these. The plotted frequency curves from these figures are shown in Chart 1.

The curves certainly display marked differences, the mode of one falling at +0.05 while that of the other falls at +0.90. The curve of the pseudodiphtheria bacillus is based upon enough figures from a sufficient number of strains to be fairly accurate. The curve of the true diphtheria organism, however, is not entirely satisfactory.

Because of the wide range of variation in the amount of acid produced by different strains of this organism, a satisfactory curve on this

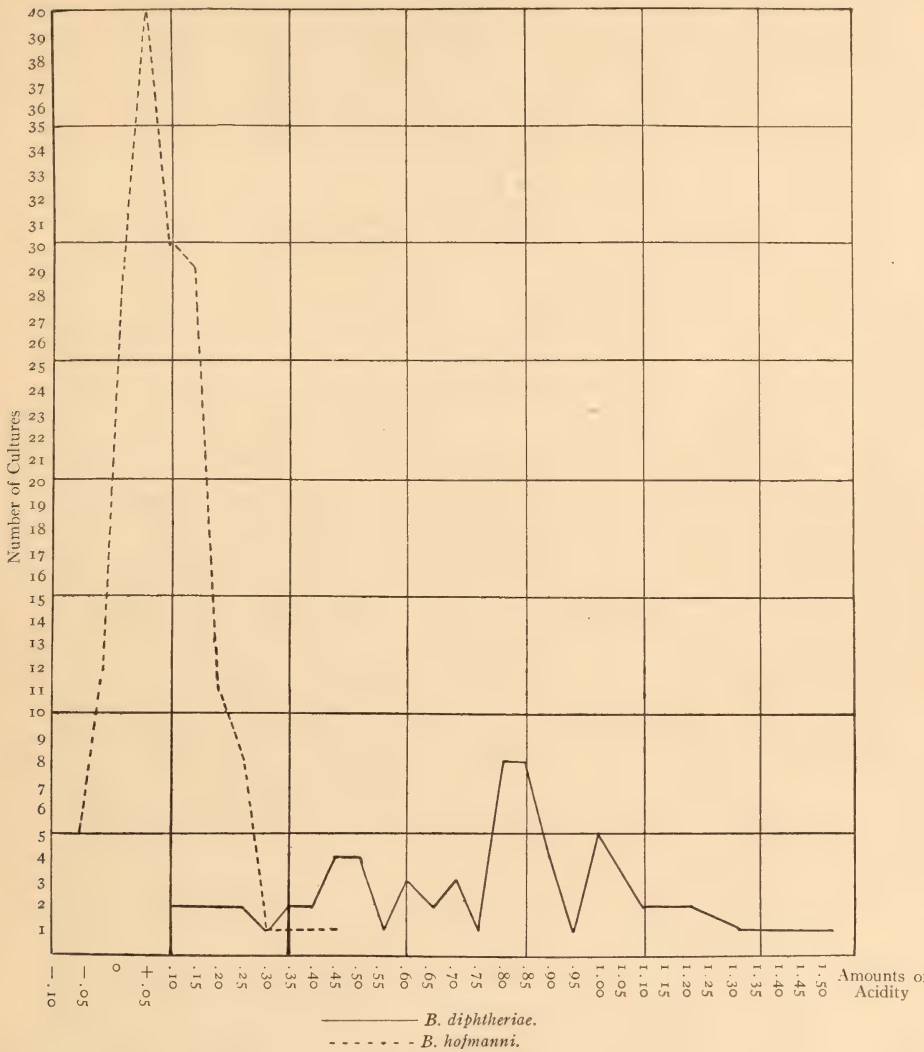

CHART 1.

scale could not be produced from less than five or six hundred pure cultures, preferably from at least one hundred separate strains.

The writer believes, however, that the curves here presented are

sufficiently accurate to show that there is a marked difference between the two organisms.

TABLE 3.

ACID PRODUCTION OF B. HOFMANNI.

Acidity	No. of Cultures	Acidity	No. of Cultures	Acidity	No. of Cultures
−0.11	1	0.03	1	0.15	17
−0.09	2	0.05	19	0.16	10
−0.08	2	0.06	11	0.20	7
−0.05	2	0.07	9	0.21	4
−0.04	3	0.09	2	0.25	7
−0.03	7	0.10	14	0.26	1
0	12	0.11	10	0.32	1
0.01	6	0.12	4	0.45	1
0.02	11	0.13	2		

Total number of cultures, 166.

TABLE 4.

ACID PRODUCTION OF B. DIPHTHERIAE.

Acidity	No. of Cultures	Acidity	No. of Cultures	Acidity	No. of Cultures
0.12	2	0.50	1	0.87	7
0.25	1	0.52	1	0.90	2
0.27	1	0.53	1	0.92	2
0.30	1	0.62	3	0.97	1
0.35	1	0.65	1	1.00	5
0.37	1	0.67	1	1.10	2
0.42	2	0.72	3	1.15	2
0.43	1	0.77	1	1.20	2
0.45	2	0.78	1	1.30	1
0.47	1	0.82	7	1.37	1
0.48	2	0.85	1	1.50	1

Total number of cultures, 62.

SUMMARY OF THE ABOVE EVIDENCE.

We may sum up the evidence as follows: The series of cultures taken during the course of the disease show no tendency, except in a few cases, toward a change to the solid-staining forms. We believe, from our observation, that the sequence of types during the development of individual cultures, together with the fact brought out by Denny, that symbiosis with a large number of other organisms inhibits the change to granular types, adequately explains any opposite results.

The successive passage of a number of strains of B. hofmanni through many guinea-pigs, animals peculiarly susceptible to diphtheria toxin, did not change the morphology nor increase the virulence of the organisms. B. hofmanni also proved to be non-virulent to canaries, pigeons, and chickens, and successive inoculations through these birds produced no effect.

Doses as large as 7 per cent of the body weight inoculated into both half-grown and very young guinea-pigs produced no change in B. hofmanni, nor did these excessive inoculations have any other apparent effect on the animals than the production of a large amount of edema at the point of inoculation.

Cultures sensitized by contact with the serum of normal guinea-pigs produced, when inoculated, no additional effects on the guinea-pigs and no change in the organisms.

A large increase of the amount of oxygen present in the atmosphere in which the cultures of B. hofmanni were incubated did not produce any unusual effects.

Three passages through guinea-pigs, constituting a total stay of 32 days, in celloidin sacs in the body cavity of a susceptible animal, produced no change in B. hofmanni. Simultaneous inoculation with B. diphtheriae and with Aurococcus aureus, both with and without celloidin sacs, also caused no metamorphosis.

Cultures of B. hofmanni were inoculated into animals, together with diphtheria toxin, both directly and in celloidin sacs, with no change in the organisms inoculated.

A series of selections of those types approaching the morphology of virulent organisms made from cultures of B. hofmanni and carried through a period of twelve "generations," even in a strain which exhibited a number of barred types in the original culture, produced no permanent change. Each strain was as typical morphologically and in its action on guinea-pigs at the end as at the beginning of the experiment.

In individual cultures of B. diphtheriae, as described by Denny and confirmed by our own work, there occurs a sequence of types ranging from the solid-staining D^2, C^2 types through the barred to the granular types. We have even found one culture of virulent B. diphtheriae which presented only D^2 types even after 16 hours' incubation, and one culture, also, which exhibited D^2 types both before and after inoculation into a guinea-pig.

In considering the morphology, it should be further borne in mind that strains of B. hofmanni sometimes show occasional barred types, and in our observation, one culture of B. hofmanni, after 65 hours' incubation, showed a considerable proportion of these seg-

mented types. Most strains, however, exhibit much smaller forms than normally, when incubated for this period.

Frequency curves of the acid production of the B. hofmanni and B. diphtheriae were markedly different. This seems to be positive evidence of specific difference between the two organisms.

DISCUSSION.

It seems to the writer that one reason for the confusion which has existed among bacteriologists in regard to these two species of bacteria may be traced to a misconception of the nature of species. There are always variations in a given species and merely because intermediate forms between one species and another can be found, it does not signify that the two are necessarily one. Particularly is this true in dealing with unicellular organisms like bacteria. We should naturally expect to find connecting forms between closely allied species.

Literally thousands of strains of pseudodiphtheria organisms have been tested for virulence only to find it lacking in all but possibly one or two cases. And as it is evident from our work that some strains of the true diphtheria organisms retain the solid-staining morphology much longer than is generally supposed, we think that most of these virulent D^2 types would have shown barred or granular morphology if they had been grown longer. If we could plot the curve of frequency of all these attempts conducted by many investigators in all parts of the world, it would present just the "mountain tops" and "valleys" we find in tabulating many admittedly different species. If only a hundred tests of the virulence of these organisms had been made, it would be the better part to suspend judgment in regard to this point until more data should be at hand. But as is well known, the number is not hundreds but thousands and is, therefore, sufficient basis for judgment.

As has been shown in this paper, the frequency curves of the action of the two organisms on dextrose are different. Here too, indeed, intermediate forms are found and some diphtheria organisms produce even less acid from dextrose than some pseudodiphtheria strains, but the modes of the curves are decidedly different.

In morphology, also, there is no question but that there is, as we

should expect, an intergrading of types between the two species. There is also little doubt, however, that in well-grown cultures the two organisms usually show distinct morphological differences.

We think that a second very important cause for confusion lies in the failure to recognize that young forms of B. diphtheriae on blood-serum cannot be distinguished from the pseudodiphtheria bacillus. The morphology of the individuals of the two species should be compared only when they have been given proper opportunities for development.

The writer would suggest that a longer incubation than the customary 15 or 18 hours would probably in almost all cases settle the question as to the identity of a given bacillus. This would give opportunity for the D^2 forms of the diphtheria bacillus to develop into longer typical forms while the D^2 forms of the pseudodiphtheria bacillus would remain the same. It must be remembered, however, that *very long* incubation, 60 hours or more, may cause some strains of B. hofmanni to depart from the D^2 type. Also the virulent pseudodiphtheria types, which produce quite distinct symptoms from the true diphtheria bacillus, and whose toxic effect is not neutralized by diphtheria antitoxin, must be kept in mind.

Should not Wesbrook's symbol of D^2 be restricted to those organisms which produce acid and diphtheria toxin and never applied to the typical non-acid-producing, non-pathogenic pseudodiphtheria bacillus? The writer would also venture to suggest that this latter name be discarded for the less cumbersome and less perplexing one of B. hofmanni.

CONCLUSIONS.

1. Solid-staining types are not more prevalent at the end than at the beginning of a case of diphtheria.

2. Successive passages of B. hofmanni through guinea-pigs, chickens, pigeons, or canaries produce no effect either on the animals or on the organisms inoculated.

3. Doses as large as 7 per cent of the body weight of half-grown or young guinea-pigs do not kill the animals nor change the type of B. hofmanni.

4. Guinea-pigs inoculated with cultures of B. hofmanni sensitized with homologous serum show no unusual effects.

5. B. hofmanni grown in an increased supply of oxygen shows no biochemical or morphological change.

6. By using celloidin sacs it was found that long-continued growth in the body cavity of guinea-pigs either alone or together with B. diphtheriae or Aurococcus aureus does not change B. hofmanni.

7. B. hofmanni, inoculated into animals in combination with toxin, either directly or in celloidin sacs, exhibits no change in the cultures recovered.

8. Artificial selection on the basis of morphology does not change the form of B. hofmanni.

9. Solid-staining forms are common to both B. hofmanni and B. diphtheriae during the first 8 to 12 hours of growth. Occasionally, however, these types are retained by the B. diphtheriae for much longer periods and some strains of B. hofmanni may show barred types on long incubation.

10. The frequency curves of acid production of B. hofmanni and B. diphtheriae show marked differences.

11. We would suggest that the term pseudodiphtheria bacillus be discarded for the less perplexing one of B. hofmanni and that the symbol D^2 be restricted to those organisms of the correct morphology which produce acid and diphtheria toxin.

12. From a careful study of the literature and from the experiments described in this paper, we are forced to take the position that the pseudodiphtheria bacillus or B. hofmanni belongs to a different species from the true Klebs-Löffler bacillus. Doubtless both organisms do belong to the same group and came from common ancestors, but the differences seem to be sufficiently constant to place them in separate species.

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PLAGUE INFECTION IN A BRUSH-RAT (NEOTOMA FUSCIPES).*

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The brush-rats are members of the same family, the Muridiae, to which the ordinary rats and mice belong; however, the genus Neotoma is classified under the subfamily, Neotominae, while the domestic rodents belonging to the genus Mus are assigned to the subfamily Murinae. In view of the well-known susceptibility to plague of members of the genus Mus, it is not surprising to find that this somewhat closely related rodent has been found infected, altho I am not acquainted with any previous report of this disease in any member of the genus Neotoma.

According to Elliot[1] Neotoma fuscipes (Baird) is found in the "Coast Region of California and Oregon, from Monterey Bay to the Columbia River." Other species of the same genus are found in various localities in the United States and in Mexico. Stephens[2] states that the type locality of Neotoma fuscipes (Baird) is Petaluma and Santa Clara, California, and that the species is "found from Monterery County north to Lake County" (California).

The following quotation relating to the habitat and to the habits of this rodent, which is generally known as the dusky-footed brush-rat, is taken from Stephens' *California Mammals:*

> Dusky-footed brush-rats inhabit the chemisal and the underbrush in open forests and groves, rarely being found in thick forests. This form does not appear to occur high in the mountains, seldom up to 3,000 feet altitude. The food is principally vegetable, but it is quite varied. They have the usual generic propensity for carrying off small articles.
>
> The breeding season is March to June, perhaps later. The number of young in a litter is two to four. The home is usually in a "nest" or "house" of sticks, twigs, bones, or anything portable, these piles of rubbish being two to four feet high, roughly cone-shaped, and are usually placed in a thicket of brush, sometimes against a tree.
>
> Occasionally the brush-rats take up their residence in barns or other buildings, where they do the most harm by carrying off small articles, stored vegetables, dried

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[1] *Synopsis of the Mammals of North America and the Adjacent Seas,* Field Columbian Museum, Chicago, 1901.

[2] *California Mammals,* The West Coast Publishing Company, San Diego, 1906.

fruits, grain, or anything they can carry off, even if utterly useless to them except to swell their rubbish pile. They seldom gnaw anything, however. They leave the premises immediately on the arrival of the introduced species of rat, which is a greater nuisance.

A small number (not over 40 or 50) of these animals have been examined at the Federal Laboratory at San Francisco, but only one presented any lesions that led to the suspicion of plague infection.

The rat in question was provisionally identified by my assistant, Mr. M. B. Mitzmain, as Neotoma fuscipes. For the confirmation of this provisional identification I am indebted to Professor Joseph Grinnell of the Department of Mammalogy of the University of California. The specimen was a full-grown male. Mention is made of the size, as a very considerable percentage of the grown domestic rats in this vicinity are immune to plague, and it may be a matter of interest at some future time to determine the susceptibility of large and small brush-rats.

Gross lesions.—The gross pathological changes were confined to the liver and to the right lung. The liver was literally crowded with fine yellowish-white granules, averaging somewhat smaller than a mustard-seed in size. The organ, aside from the granules, was rather ashen in color. The granules were similar to those often seen in plague in ordinary rats, but were larger and more prominent. They could not possibly have been overlooked by anyone familiar with the pathology of rodents, and in fact they at once attracted the attention of the laboratory assistant who was dissecting the animal.

The right lung was partially consolidated, dark red in color, and somewhat friable.

The spleen was apparently enlarged, but as we had no normal brush-rat for the purpose of comparison, no definite statement can be made on this point. It has been my experience that among presumably healthy rodents there is a great variation in the size of the spleen.

It is noteworthy that there was no bubo. A careful search was made for lesions of the lymph glands, but none was found.

The smears from the liver and from the spleen were negative; that is to say, they showed no organisms bearing any close resemblance to the pest bacillus. A smear from the consolidated lung tissue showed an enormous number of spherical bodies which agreed in size and in shape with the "coccoid" forms of B. pestis. A few fairly well-defined bipolar forms were also present. The organisms in general were grouped as if clumped, and not uniformly distributed over the field, as is usually the case in smears from plague tissue. All of these organisms were found to be decolorized when treated by Gram's method.

Cultures were not made from the organs of the animal as the smears showed that several organisms were present; therefore it was considered better to resort to the more certain method of animal

inoculation for the purpose of establishing a diagnosis. Two guinea-pigs were inoculated subcutaneously with tissues from the brush-rat. One guinea-pig was inoculated with lung tissue and died at the end of five days with lesions of acute plague. Cultures were contaminated. The second animal, inoculated with liver tissue, died at the end of five days with lesions of acute plague, and a pure culture of B. pestis was obtained from heart blood and liver.

It should be stated here that this experiment does not prove that plague bacilli were present in both of the tissues used for inoculation as the same instruments were employed throughout the dissection of the animal and the subsequent inoculations and it is possible that bacilli may have been present in one organ only (lung) and have been carried mechanically to the other.

The lesions in the guinea-pigs were entirely characteristic of plague. The organism isolated from the heart blood and from the liver gave a typical growth upon agar and in broth; upon 3 per cent salt agar the usual involution forms were developed in 24 hours. Litmus milk was unchanged even after two weeks' observation (difference from B. pseudotuberculosis rodentium [Pfeiffer]).

The result of the inoculation of the guinea-pigs made it desirable to carry out further experiments in order that the diagnosis might be placed upon as firm a foundation as possible. The next experiment was carried out with tissue that had been preserved in the ice-chest.

TABLE 1.

Animals	Mode of Inoculation	Day of Death	Lesions	Cultures
Guinea-pig............	Cutaneous	7	Acute plague	Pure culture of B. pestis from heart blood and kidney
White rat..............	Cutaneous	5	" "	Pure culture of B. pestis from heart blood and spleen
White rat..............	Subcutaneous	Killed on 11th day	Chronic "	None made

The infection of a guinea-pig and of a white rat by the cutaneous method of inoculation furnished additional and very strong evidence in favor of the infecting organism being the plague bacillus; however, it seemed wise to determine the protective power of anti-pest serum against it.

RELATION TO ANTI-PEST SERUM.

No doubt was entertained now as to the nature of the organism, but as this was believed to be the first case of plague in a member of the genus Neotoma, we wished to make the evidence as convincing as possible. The full record of the work with the anti-pest serum is given, for altho it is realized that some of the results are of little value for the present purpose, they serve to illustrate some of the uncertainties in these procedures.

Series 1.—The specimen of anti-pest serum used in the first experiment was over two years old and the outcome of the test seems to indicate that it had but little protective power.

In each case the animal was vaccinated with the spleen of one of the white rats mentioned in the preceding experiment. This rat had died on the fifth day, and the spleen showed large numbers of pest-like bacilli. A pure culture of the organism was isolated from that organ. In this and in the subsequent experiments, the serum was given intraperitoneally immediately before the inoculation with the infecting organism. In each case the infecting organism was rubbed into the shaven belly.

TABLE 2.

Animal	Weight Grams	Serum Intraperitoneally	Day of Death	Lesions	Cultures
Guinea-pig.....	390	4 c.c. normal horse (control)	6	Acute plague	Pure culture of B. pestis isolated from kidney
" "	410	4 c.c. anti-pest	6	" "	Pure culture of B. pestis isolated from liver
White rat......	150	4 c.c. normal horse	5	" "	Pure culture of B. pestis from heart blood
" "	145	4 c.c. anti-pest	Killed on 11th day	Chronic "	None made
" "	200	4 c.c. normal horse	5	Acute "	" "
" "	130	4 c.c. anti-pest .	Killed on 11th day	None	" "
" "	105	4 c.c. normal horse	4	Acute plague	Contaminated
" "	130	4 c.c. anti-pest	5	Doubtful	"

In this case the serum had no influence upon the course of the disease in the "protected" guinea-pig. Two of the three "protected" rats survived; when killed one presented no lesions; the other had a purulent gland, which was interpreted as a lesion of chronic plague. The third "protected" rat died on the fifth day. The lesions were doubtful, and, unfortunately, the culture was contaminated. The

control rats all died of acute plague. Upon the whole, it may be said that this experiment no more than indicates that the serum exerted a considerable protective action upon the white rats, saving two out of three.

Series 2.—In the next experiment a comparatively fresh specimen of serum was used. The infecting material was a four-day-old broth culture isolated from the white rat (see Table 2) which had succumbed on the fifth day after cutaneous inoculation. The culture was the one derived from the same rat, the spleen of which furnished the infecting agent for the preceding experiment. The results are shown in the following table:

TABLE 3.

Animal	Weight Grams	Serum Intraperitoneally	Day of Death	Lesions
Guinea-pig	295	2 c.c. normal horse (control)	6	Acute plague
" "	270	2 c.c. anti-pest	12	Chronic "
" "	270	2 c.c. anti-pest	6	Acute "
White rat	70	2 c.c. normal horse (control)	Killed 13th day	Abscess at site
" "	80	2 c.c. anti-pest	Killed 13th day	No lesions

This experiment would lead one to conclude that anti-pest serum had practically no influence upon the course of the infection with the organism under consideration, altho one of the "protected" guinea-pigs lived twice as long as the control.

I am unable to say why the control white rat failed to succumb. There is no reason for believing that normal horse serum exerts any protective power against B. pestis.

Series 3.—A third and final series of inoculations was made, using a sample of anti-pest serum still more recent than that used in the preceding experiment. The results are shown in the next table. A

TABLE 4.

Animal	Weight Grams	Serum Intraperitoneally	Day of Death	Lesions
Guinea-pig	350	3 c.c. anti-pest	Killed on 35th day	No lesions
" "	433	" " "	11	Subacute plague
" "	380	Control, no serum	5	Acute "
" "	220	" " " "	4	" "
White rat*	...	3 c.c. anti-pest	Killed on 16th day	None
" "	...	" " "	" " " "	"
" "	...	" " "	" " " "	Small purulent focus in axillary gland
" "	...	Control, no serum	4	Acute plague
" "	...	" " "	6	" "
" "	...	" " "	6	" "

* The white rats were not weighed, but were all of approximately the same size.

broth suspension of a 48-hour agar culture isolated from one of the guinea-pigs in an earlier experiment was used as the infecting agent. The animals were all inoculated by the cutaneous method.

This experiment was entirely satisfactory in demonstrating the protective influence of the serum. One protected guinea-pig survived while the other markedly outlived the controls. The protected white rats survived, while the controls all died of acute plague.

GENERAL CONSIDERATIONS.

The question as to how the brush-rat was infected is one that cannot be answered now. Whether it was from another case of plague among its own species, or whether it was infected from a ground squirrel, or some other rodent, is purely a matter of conjecture. The rodent came from a county (Alameda) in which many infected squirrels have been found.

It would be idle to attempt to point out any special significance in the finding of plague in one of these rodents. It is perhaps worth while to call attention to the statement in the quotation from Stephens that "occasionally the brush-rats take up their residence in barns or other buildings." It is possible that by frequenting the habitations of man they may occasionally be the source of plague infection in human beings, either directly or through the ordinary house-rats.

The most that can be said at present is that the fact, now established, that these animals may have plague adds another factor to the already perplexing problem of the transmission of plague among the rodents of the Pacific Coast. Fortunately, the brush-rat is not sought for its flesh, as is the ground squirrel, nor does it ordinarily build its nests near human habitations as do the domestic rats.

THE SUSCEPTIBILITY TO PLAGUE OF THE PRAIRIE DOG, THE DESERT WOOD RAT, AND THE ROCK SQUIRREL.*

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The animals (other than controls) used in this experiment were trapped upon the U.S. Marine Hospital Reservation at Fort Stanton, New Mexico. After they had been kept in captivity for several weeks an effort was made to determine the susceptibility of the several species to infection with the plague bacillus.

The culture of B. pestis used was one that had been isolated from the spleen of a naturally infected ground squirrel about two months prior to the present experiments. The third generation on agar was used, the culture having been grown for 72 hours at 37° C.

In the case of the animals in which the culture was inoculated subcutaneously, one loopful of the agar culture was suspended in 100 c.c. of salt solution (0.8 per cent), and a volume of the suspension sufficient to contain the dose shown in the table injected beneath the skin of the belly. When the cutaneous method of inoculation was practiced, a loopful of undiluted agar culture was rubbed into the shaven skin of the belly.

The dose used subcutaneously was large enough in each case to infect a susceptible animal with certainty, but was not large enough to make it necessary to take the question of plague intoxication into account. The question of intoxication does not need to be considered in the cutaneous inoculations.

For the purpose of controlling the dose and the virulence of the culture, we have used, as will be seen from an examination of the table, a guinea-pig and three black rats (Mus rattus). It will be seen by a glance at the table that the rats died in about the usual time that one expects a plague-infected rat to die.

The guinea-pig sickened promptly, developed a large brawny reaction at the site of inoculation, and showed marked enlargement

* Received for publication February 12, 1910.

of the inguinal lymphatic glands. The symptoms abated, however, and the animal recovered, showing no lesions of plague when it was chloroformed on the twenty-fifth day after inoculation. Other guinea-pigs inoculated from the same culture have succumbed to plague in the usual time and, so far as we are able to judge, the failure of the culture to kill this particular rodent was due to the presence of a considerable degree of natural immunity. Natural immunity of guinea-pigs to plague while rarely encountered is recognized by nearly all who have studied the relations of these rodents to plague infection.

The following table gives all of the data in regard to each animal:

Animal	Weight Grams	Dose of Culture	Day of Death	Lesions	Remarks
Rock squirrel......	...	0.01 loop	4	Acute plague	
Rock squirrel......	320	0.005 "	4	" "	
Rock squirrel......	385	0.001 "	5	" "	
Rock squirrel......	600	"Vaccinated"*	3	" "	
Rock squirrel......	560	" *	5	" "	
Wood rat.........	170	0.01 loop	3	" "	
Prairie dog........	1,100	" "	6	" "	
Guinea-pig	400	" "	Killed 25th day	None	Control, animal sickened but recovered, probably naturally immune
Black rat..........	135	" "	4	Acute plague	Control
Black rat.	60	0.001 "	4	" "	"
Black rat	?	"Vaccinated"*	4	" "	"

* Cutaneous inoculation.

A culture of B. pestis was isolated from the heart blood or from the liver or spleen of all the animals that died.

The statement in the column headed "lesions" should be qualified by the explanation that as the wood rat and the prairie dog were the only members of these species we have ever seen infected with plague there was no standard with which to make a comparison but the lesions were similar to those seen in other rodents that have died of acute plague.

In regard to the nature of the lesions, we may say that they presented no points of special interest with the possible exception of the fact that careful search failed to show any evidence of a bubo in the prairie dog. The wood rat presented changes similar to those seen in ordinary rats artificially infected. In the rock squirrels the findings were identical with those seen in naturally and artificially infected ground squirrels (Citellus beecheyi).

It might perhaps have been assumed in advance that all of these animals would prove susceptible to plague infection, in view of the fact that closely related species are known to be very susceptible to the infection.

The results of the work may be summarized as follows:

The rock squirrels are quite readily infected, probably being equally as susceptible as the ground squirrel (Citellus beecheyi).

As but one prairie dog and one desert wood rat were available it would be unsafe to go farther than to assert that the specimens tested exhibited no evidence of immunity to plague; on the other hand, judging by this one experiment they appear to be quite susceptible to the infection.

The scientific and common names of the rodents mentioned, their place in the natural order to which they belong, their relation to one another and to species that are known to contract plague in nature are shown here:[1]

Order, *Glires*
- Family, *Scuridae*
  - Subfamily, *Scuirinae*
    - Genus, *Citellus*
      - Species, *Citellus grammurus*, Say (Rock squirrel)
      - (N.I.) Species, *Citellus beecheyi*, Richardson (California ground squirrel)
  - Subfamily, *Marmotinae*
    - Genus, *Cynomys*
      - Species, *Cynomys ludovicianua arizonensis*, Mearns (Arizona prairie dog)
- Family, *Muridae*
  - Subfamily, *Neotominae*
    - Genus, *Neotoma*
      - Species, *Neotoma albigula angusticeps*, Merriam (Eastern desert wood rat)
      - (N.I.) Species, *Neotoma fuscipes*, Baird (Dusky footed wood rat)
  - Subfamily, *Murinae*
    - Genus, *Mus*
      - (N.I.) Species, *Mus rattus*, Linnaeus (Black rat)
      - (N.I.) Species, *Mus norvegicus*, Erxleben (Brown rat)
      - (N.I.) Species, *Mus musculus*, Linnaeus (House mouse)
- Family, *Caviidae*
  - (N.I.) *Cavia cobaya*, Pallas (Guinea-pig)

The mammals found infected in nature are indicated by N.I.

[1] We wish to express our indebtedness to Professor Joseph Grinnell of the Museum of Vertebrate Zoölogy of the University of California for the accurate zoölogical identification of these rodents and for assistance in preparing the classification.

THE DISTRIBUTION OF BACTERIA IN BOTTLED MILK AND CERTAIN CONTROLLING FACTORS.*

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It has been known for some time that the cream of cow's milk is always much richer in bacteria than the leaner portions, but as Anderson[1] has remarked and as his review of the scanty literature shows, the fact of this unequal distribution has attracted very little attention. The results of recent investigations of Hess[2] and later of Anderson, however, suggest that this excessive bacterial contamination of the top milk may be of some import in the etiology of infantile summer intestinal disturbances, as it is a common practice to use this portion of bottled milk in the preparation of modified milk for bottle-fed infants. Anderson has found in an examination of a large number of samples of Washington milk that both gravity and centrifuged cream contain from 10 to 500 times as many bacteria as the mixt milk.

With the purpose of mitigating in a measure the possible danger to infants of ingesting such excessive numbers of bacteria, Hess[3] has advised that the first two ounces from the bottle of milk be removed and set aside for table use, and that the following 7, 8, or 12, ounces (giving respectively 12 per cent, 10 per cent, and 7 per cent cream) alone be used in infant feeding. He believes that this procedure is justified through his finding that the top layers of cream have a higher bacterial content than the subjacent portion.

In this paper are embodied the results of the examination of a large number of quart bottles of milk (over 90) which were in some degree representative of the average bottled milk supply of New York City during the past summer, ranging in quality from that supplied by

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[1] *Jour. Infect. Dis.*, 1909, 6, p. 392.

[2] *Archives of Pediatrics*, 1908, p. 31.

[3] *Jour. Amer. Med. Assoc.*, 1909, 53, p. 523.

shops on the "East Side" to the highest quality of certified milk. This work was undertaken at the suggestion of Dr. Joseph E. Winters and with the aim of determining:

a) In greater detail the relative distribution of bacteria in bottled milk especially as regards the cream layers;

b) To what degree the bacterial count is decreased by following the procedure of Hess;

c) What are the more important factors which influence the disposition of the bacteria in the bottle.

Methods.—The bottles of milk were obtained either from many sources or at times from a single dairy, as best served the purpose of the experiments. After delivery at the laboratory these bottles had stood for two hours or more in the ice-box before sampling so that there was always a distinct line of demarkation between the cream and the skim milk.

For plating, dilutions of the milk were made in sterile normal salt solution in multiples of ten. At each step the test tubes were shaken vigorously and stirred with the pipette to break up the clumps of bacteria which are so frequently present in milk and which, left intact, give a false impression as to the degree of bacterial contamination. In each instance 1 c.c. of the dilution was plated. The media, with the exception of the litmus lactose agar, was titrated to 1.5 acid to phenolpthalein as recommended by the Committee on Bacterial Milk Analysis. In the majority of the experiments meat infusion peptone broth served as the basis for the agar and the gelatin, altho in the earlier work the nutrient agar was prepared with Liebig's extract of beef. A comparative series disclosed a slightly lower average count with the "extract" agar, and hence it was discarded. Agar of the percentage of 1.2 and gelatin of 10 were used.

The agar plates were incubated for 24 hours at 37° C., and the gelatin, for three days at room temperature. With certain of the agar plates a supplementary incubation at room or 27° C. temperature was given. As has been shown by Wilcox[1] and others, a higher average count is obtained by the longer incubation at the lower temperature, but as the increased growth was quite evenly distributed through the cream and the skim milk, for the purpose of these experiments the shorter incubation at 37° C. served fully as well.

Samples from different parts of the milk bottle were obtained in several different ways. In the first series the cruder procedures were followed, which give an insight into the numbers of bacteria in milk as commonly fed to infants. In the second series smaller samples were obtained with less disturbance of the milk.

1. Samples obtained with a 50 c.c. pipette.—This mode of sampling was used in a series of 10 bottles of milk of various degrees of contamination. The first four samples were removed consecutively (the first three consisting entirely of cream and the fourth of cream

[1] *Studies from the Research Laboratory, Department of Health, City of New York*, 1907, 3, p. 186.

with a small admixture of skim milk). The fifth sample consisted of the middle skim milk and the sixth of the sediment portion.

2. Sampling with an ounce milk dipper.—In a series of 12 bottles of ordinary milk from various dairies in the city the fluid was dipped out ounce by ounce with an ounce milk dipper, using no greater caution against mixing and stirring the fluid than would be taken in the everyday preparation of an infant's diet. From four to six consecutive ounces were dipped from the top of the bottle, one from the middle and one from the sediment portion. In certain instances all of the dipped samples were added to the remaining milk and the whole mixt and plated to determine the bacterial count for the "whole milk."

TABLE 1.

Distribution of Bacteria in Bottled Milk.

Samples	Samples Obtained with a 50 c.c. Pipette			Samples Obtained with a 1-Ounce Milk Dipper		
	Average of 10 Experiments	Average of 5 Low-Count Bottles	Average of 5 High-Count Bottles	Average of 12 Experiments	Average of 5 Low-Count Bottles	Average of 7 High-Count Bottles
Top	827,000	147,500	1,523,700	4,745,000	509,200	7,771,000
Second	964,500	101,000	1,828,000	5,397,000	552,500	8,857,000
Third	596,700	17,400	1,177,600	2,918,000	413,800	4,707,000
Fourth	270,440*	19,750	468,400	2,493,000	302,900	4,043,000
Fifth	....	....	....	2,470,000†	....	....
Sixth	....	....	....	1,960,000†	....	....
Middle skim milk	17,850‡	17,500	....	428,000	112,000	654,000
Sediment	153,850	21,400	286,800	521,000	131,675	819,000

* Nine experiments only.

† Four experiments only.

‡ Six experiments only.

3. Samples poured from bottle.

TABLE 2.

Samples Obtained by Pouring or Dipping Various Amounts of the Milk.

Samples	First 2 Ounces Poured off and Following Samples Dipped with 1-Ounce Dipper		Sampled by Pouring. Each Sample 1 Ounce	Sampled by Pouring. Each Sample 2 Ounces
	Average 5 Experiments	Exceptional Count, not Averaged	Average 3 Experiments	Average 5 Experiments
1. First 2 ounces	1,025,000	730,000	284,000 137,000	460,000
2. Third ounce	1,434,000	9,100,000	64,000	116,000
3. Fourth ounce	1,288,000	2,300,000	120,000	
4. Fifth ounce	1,204,000	500,000	....	90,000
5. Sixth ounce	454,000	320,000	....	
6. Skim milk	67,000	200,000	17,000	36,000

The following chart illustrates graphically the very marked difference in the degree of the bacterial contamination in the cream layers as compared with the skim milk. In this chart the count for the middle skim milk is used as the unit of measure and the ordinates express the ratio of the bacterial content of other portions of the

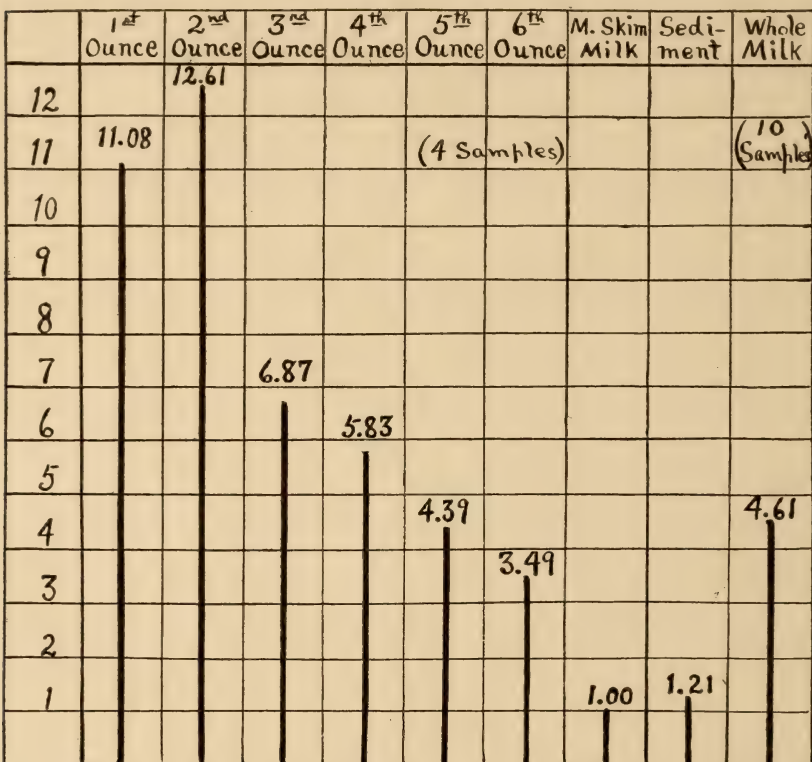

CHART I.—Showing the difference in the degree of contamination of various cream layers and the skim milk.

bottle and of the mixt milk to this unit.[1] If more grossly contaminated milk had been used as the basis of this chart, without doubt the differences in the length of the lines would have been much greater, but it seems probable that it expresses fairly accurately the disposition of bacteria in the average bottle of fresh milk with a count of about 1,000,000 bacteria per c.c.

[1] Unless otherwise stated the ratios were determined from the average count of 12 samples of medium grade milk.

The general average of these pouring and dipping experiments disclosed a preponderance of bacteria in the second ounce of cream over the first. On the contrary the counts given by Hess show this relation regularly reversed in that the first ounce of cream embodied on the average over 100 per cent more bacteria than the second. Without doubt this discrepancy is due to the fact that according to his tables the samples of milk examined by him contained in general fewer bacteria than did ours. We have found that when the upper cream averaged under two million bacteria per c.c. the conditions outlined by Hess obtained, but when the count was much over that mark the second ounce often revealed a 100 per cent higher bacterial content than the first.

These first two ounces, however, as Hess has maintained, frequently contain many more bacteria than the lower layers of the cream, for with the third ounce there is generally a marked diminution in the count which continues as we approach the skim milk. At times, nevertheless, one encounters a striking exception to this generalization, a good example of which is given in the second column of Table 3. In this bottle the third ounce averaged over 12 times the count of the first two ounces.

4. Samples obtained with a 1 c.c. pipette.—In dipping samples from the bottle the fluid is unavoidably more or less disturbed with a consequent redistribution of bacteria. Samplings with a 1 c.c. pipette, carefully performed, give a truer picture of the actual bacterial contamination of the various strata. The results, however, are of more theoretical than practical interest.

The various depths at which samples were desired were indicated by marks on a sterile pipette, measured from the tip. After rinsing with sterile salt solution, and with the thumb tightly pressed over the mouth end, the pipette was sunk vertically into the fluid until the surface reached the measured mark. The milk was then drawn up in a little excess of 1 c.c., the pipette removed and its exterior thoroughly rinsed off. Discarding the excess, the 1 c.c. sample was diluted and plated. Experience indicated that this is a satisfactory and reliable method for the purpose of the experiment.

The results with this method of sampling are in accordance with those obtained by dipping, in that they reveal an excess of bacteria

in the upper third of the cream, as compared with the lower portions. The samples from the surface layer, constituting what might be

TABLE 3.

DISTRIBUTION OF BACTERIA IN BOTTLED MILK. SAMPLES OBTAINED WITH A 1 C.C. PIPETTE.

Average Bottled Milk			Certified Milk			Exceptionally "High Counts"	
Samples	Average of 7 Experiments	Ratio to Sample 6 Top Skim	Samples	Average of 13 Experiments	Ratio to Sample 5 Middle Skim	Average of 2 Experiments	Ratio to Sample 5 Middle Skim
Cream:							
1. Surface	510,000	17.00	1. Top cream...	22,700	51.49	11,960,000	2.40
2. ½ inch......	406,000	13.53	2. Middle cream	15,370	34.93	6,860,000	1.38
3. 1 inch......	288,000	9.60	3. Bottom cream	10,390	23.61	3,900,000	.78
4. 2 inches	310,000	10.33	4. Top skim....	1,030	2.34	3,900,000	.78
5. 3 inches	164,000	5.46	5. Middle skim .	440	1.00	4,970,000	1.00
Top skim milk:							
6. 4 inches....	30,000	1.00	6. Sediment	320	.72	2,400,000	.48
Sediment:							
7. 7 inches....	18,000	.60					

called the scum of the cream, show the maximum counts, and as the samples approach the lean milk here is a gradual but frequently irregular drop in the degree of contamination.

PRACTICABILITY OF DISCARDING THE FIRST TWO OUNCES OF CREAM.

These various methods of sampling have shown that, as Hess contends, there is not a homogeneous distribution of bacteria in the cream, but the greater numbers are, on the average, in the top layers. In Table 4 is indicated the degree to which milk, for infant feeding, is improved bacteriologically by discarding the upper two ounces. These figures are not absolutely correct but are sufficiently so for the purpose in view. The error is always toward the side of indicating greater reduction than actually occurs. In each instance the cream is calculated as six ounces.

By discarding the first two ounces of cream and utilizing the next eight ounces (10 per cent cream) instead of the first eight ounces, we find in a series of 35 quart bottles of various grades of milk, sampled by four different practical and necessarily crude methods, that there occurs a decrease of 29 to 51 per cent in the bacterial content. It seems questionable if this degree of diminution is sufficient

to be of service in the purification of milk for infant feeding. The cream of New York City bottled milk contains during the hot summer weather a probable average of over 5,000,000 bacteria per c.c. A reduction of between one-third and one-half in this contamination would not materially improve the chances of the infant against the intestinal disturbances associated with an impure milk, nor would it approximate in more than negligible degree the desirable limit of bacterial contamination in milk for infant feeding which Coit has

TABLE 4.

PERCENTAGE OF DECREASE OBTAINED BY DISCARDING THE TWO TOP OUNCES OF THE CREAM.

Method of Sampling	Average Bacteria per c.c. First 8 Ounces	Average Bacteria per c.c. in the 8 Ounces Below the First 2	Percentage of Decrease According to Hess's Method
1. Pipetting—50 c.c.	(First 250 c.c.) 535,000	(First 50 c.c. discarded) 373,000	31 per cent
2. Pouring—1 ounce	94,950	54,500	46 " "
3. Pouring—2 ounces	92,950	69,500	25 " "
4. Dipping—1 ounce; 1 to 4 consecutive	High 4,920,000	2,514,000	49 " "
	Low 363,000	235,000	36 " "
5. Dipping—1 ounce; 1 to 6 consecutive	3,539,000	1,743,000	51 " "
6. Pouring first 2 ounces and dipping the others	820,000	581,000	29 " "

placed at 10,000 per c.c. If milk had become infected with B. typhosus at the farm or dairy the greater number of the bacilli would without doubt be found at the start in the upper two ounces of the cream, but by the time the milk had reached the consumer sufficient multiplication would probably have taken place to render the lower portion of the cream quite as dangerous as the upper. It is possible, however, that the danger from bovine tuberculosis might be mitigated in some degree by following the recommendation of Hess. The milk, in this instance, receives the primary infection at the time it is drawn from the cow, and as, in all probability, there would be no subsequent multiplication of the tubercle bacilli in the milk a considerable excess of them would be carried up and embodied in the upper layers of the cream. Hence the chances of infection might be somewhat greater when this top portion is utilized, than with the lower cream or the mixt milk.

FACTORS INFLUENCING THE DISTRIBUTION OF BACTERIA IN BOTTLED MILK.

MECHANICAL ACTION OF RISING CREAM.

The "rafting" activity of rising cream is, of course, the prime factor in effecting the higher degree of contamination of top milk. A number of experiments were carried out to determine the degree of effectiveness of the rising fat globules in entangling and filtering out bacteria from skim milk and to what extent the lean milk is purified in the process.

In one set of experiments a bottle of milk, in which the cream had completely separated, was sampled by the 1 c.c. pipette method at six depths (top, middle, and bottom cream and the same layers for skim milk). The proper dilutions were plated in agar, in gelatin, and in litmus lactose agar. Next the entire content of the bottle was poured into a flask and thoroughly mixt. A sample of this whole milk was plated in each medium. It was then poured back into the bottle and placed at 5° C. Platings were made at the various intervals designated in Table 5. The samples were taken at the same levels as those obtained before the shaking, these levels having been indicated by marks on the pipette. In this tabulation the ratio of change in the count (increase or decrease) from that of the mixt milk control is given for each period and media instead of the actual number of bacteria. In this way not only is space economized but a clearer impression is conveyed of the movements of the bacteria in the bottle through the agency of the cream.

Table 5 indicates that, five hours after the shaking, the bacteria have resumed practically the same distribution in the milk bottle which they held at the beginning of the experiment. A noteworthy difference, however, is present in the fact that the top cream has become proportionally richer in bacteria. It is likely that this local increase is due to the fact that the milk contains now more bacteria than at the primary rising of the cream, and hence a greater proportion would be rafted upward and incorporated in the first cream layers. The 24-hour plating shows that there has occurred a further bacterial contamination of the cream in correlation with a continued purification of the skim milk. A certain amount of this increase in the cream at 24 hours may be the result of multiplication, especially of

the lactic acid bacteria. All tendency toward sedimentation has been more than neutralized by the lifting properties of the cream, as is clear from the fact that the bottom of the bottle after 24 hours contains a smaller average number of bacteria than before it was shaken.

TABLE 5.

REDISPOSITION OF BACTERIA IN BOTTLE AFTER SHAKING MILK.

Whole (shaken) milk { Agar, 143,000 bacteria per c.c.; Gelatin, 130,000 bacteria per c.c.; Lit. lact. agar, 39,000 bacteria per c.c.

Samples (with 1 c.c. Pipette)	Before Shaking Ratios to Counts with Whole Milk			½ Hr. after Shaking	1 Hr. after Shaking			2 Hrs.	5 Hrs.			24 Hrs.		
	Agar	Gelatin	Litmus Lactose Agar	Agar	Agar	Gelatin	Litmus Lactose Agar	Agar	Agar	Gelatin	Litmus Lactose Agar	Agar	Gelatin	Litmus Lactose Agar
1. Top Cream	2.54	4.33	3.66	.38	.73	1.30	1.59	3.63	3.63	3.77	6.15	4.54	7.92	8.46
2. Middle cream..	2.93	3.92	4.66	.36	.52	.84	1.20	1.53	2.44	2.92	3.66	2.51	6.30	5.89
3. Bottom cream..	2.44	2.61	3.33	.28	.49	1.00	.97	.55	2.51	2.77	3.00	2.93	5.46	6.66
4. Top skim milk....	.25	.28	.28	.55	.54	.89	1.07	.72	.33	.23	.30	.12	.19	.21
5. Middle skim milk	.04	.11	.15	.53	.58	.87	1.05	.49	.35	.20	.33	.08	.20	.18
6. Bottom skim milk	.10	.13	.11	.51	.51	.63	1.02	.28	.12	.12	.20	.03	.05	.05

In this and other similar experiments there occurred a reduction of one-half to one-third in the average count one-half hour after shaking. It seems probable that this apparent decrease may be largely the result of clumping of the bacteria rather than an actual destruction. After one hour there was no distinct cream layer and only a slight increase of bacteria at the surface. It is, in fact, only when the cream layer begins to form that we find an excess of bacteria on the surface. That they collect on the top exactly *pari passu* with the fat globules was especially noticeable in two hours when the top layer of cream contained per c.c. several times the number in the skim milk and actually more than were present in this layer before the shaking.

COMPARISON OF THE CHANGE IN POSITION OF BACTERIA IN WHOLE MILK CONTAINING VARIOUS PROPORTIONS OF CREAM.

About one-fifth of the fluid in the average bottle of dairy milk is cream. The object of the following experiment was to determine

whether cream in less or greater ratio than this filtered out a proportional ratio of bacteria, and further to ascertain whether milk bacteria tend to collect at the surface of skim milk devoid of cream.

A bottle of fresh dairy milk was placed in the ice-box for three or four hours. The cream was then pipetted off and heated in a water bath at 65° C. for one hour. The remaining unheated skim milk was next poured into a large globular separating funnel and placed at 5° C. over night as was also the partially sterilized cream. Next morning the skim milk was drawn off very carefully and collected practically free from cream. After shaking it thoroughly a sample was plated in plain agar, gelatin, and litmus lactose agar. The partially sterilized cream was subjected to the same procedure. The mixtures of these two fluids described in the heading of Tables 7 were then made in 100 c.c. amounts and each placed in a sterile glass-stoppered bottle of that capacity. In addition, two bottles were filled with 100 c.c. of the contaminated skim milk alone, to serve as controls. The several bottles were shaken thoroughly and placed at 5° C. Platings were made with three media at the time intervals indicated in the following table. From each bottle samples of 1 c.c. were taken from the top, the middle, and the bottom. The ratios of change in this table were calculated by dividing the number of bacteria in each sample at the various periods by the average number present in each bottle at the beginning of the experiment, determined theoretically on the basis of the proportions of cream and skim milk in the several mixtures.

Table 6 indicates that the extent of the primary rise of bacteria in whole milk is correlated with the measure of its cream content With "mixture 4" a much higher proportion of bacteria were present in the two-hour cream than with mixtures 1 or 2. This is as one might anticipate, as in the former instance the fat globules, being much more numerous, are closer together and hence would entangle and raft upward a larger proportion of bacteria. At the end of two hours, when from one-half to three-fourths of the total cream has risen, these differences are especially marked. In not all of the experiments of this character was this point brought out as clearly. The degree of contamination of the skim milk is a factor of importance; when this is high the differences between the skim milk and the cream would be much less. At 5 and 24 hours the average of the ratios calculated for each of the three media is placed in the table.

In these experiments no special type of bacteria, such as those growing best at room temperature, the gelatin liquefiers, and the lactic-acid formers, exhibited any special predilection for the cream layer. In fact, after 24 hours, there was a higher average percentage of the gelatin liquefiers at the middle and bottom of the bottle than at the top. Taken all in all, however, their position as well as that

of other types of milk bacteria seems to be quite fortuitous. It may be concluded then that, of the number of pathogenic bacteria which gain access to the milk before it is finally bottled, approximately the same proportion would be carried up and incorporated in the cream as is indicated in the table for the harmless saprophytes.

TABLE 6.

INFLUENCE OF VARIOUS AMOUNTS OF CREAM ON THE DISTRIBUTION OF BACTERIA.

Mixture 1. Partially Sterilized Cream 10 c.c., Skim Milk (Contaminated) 90 c.c. = 1–10.
" 2. " " " 15 " " " " 85 " = 1–6.6.
" 3. " " " 20 " " " " 80 " = 1–5.
" 4. " " " 25 " " " " 75 " = 1–4.
Control 1. No Cream, Skim Milk 100 c.c.
" 2. " " " " 100 "

	2 HOURS	5 HOURS	24 HOURS		
	Ratios to Preliminary Counts. Agar	Ditto. Average of Ratios with 3 Media	Ditto. Average of Ratios with 3 Media	Percentage of Liquefiers	Ratio of Increase of Liquefiers
Mixture 1:					
Top	2.81	4.50	6.41	21.1	6.33
Middle	1.97	.60	3.77	28.2	1.83
Bottom	1.52	1.14	1.56	49.1	4.66
Mixture 2:					
Top	6.85	7.00	23.23	19.7	12.50
Middle	3.03	.59	.96	37.5	2.00
Bottom	2.37	.47	5.74	18.0	3.00
Mixture 3:					
Top	10.43	10.14	12.31	18.9	8.33
Middle	1.27	.50	.85	33.3	1.33
Bottom	1.27	.69	1.00	43.3	2.18
Mixture 4:					
Top	17.49	10.41	28.12	26.6	20.00
Middle	1.93	.77	.99	41.1	1.16
Bottom	2.09	1.18	1.50	13.9	.83
Control 1:					
Top	1.95	1.47	3.13	20.	3.66
Middle	2.99	.89	2.21	31.8	5.83
Bottom	1.84	1.56	7.71	30.	10.00
Control 2:					
Top	2.41	.97	1.80	31.2	5.83
Middle	2.58	2.19	9.44	40.	6.00
Bottom	2.75	2.17	4.98	30.8	13.33

The skim-milk controls of these experiments have not shown a constant predominance of bacteria on the surface. In fact, as a general rule, the greater number are to be found in the middle or the bottom of the bottle, indicating that the fat globules of the cream are the agents which dominate the peculiar distribution of bacteria in whole bottled milk. It may be mentioned in passing that the rate of multiplication of the bacteria in the lower portion of the milk is

considerably more rapid at low temperatures, at least, in the absence of a cream layer, possibly because of a freer access to the air oxygen.

INFLUENCE OF VARIOUS TEMPERATURES ON THE RELATIVE BACTERIAL CONTENT OF CREAM AND SKIM MILK.

In the comparative examination of a large number of bottles of milk, one finds that the ratio of the average count of the skim milk to that of the cream varies greatly. In certain samples the cream may contain over one hundred times as many bacteria as a like amount of skim milk, whereas in others the ratio of difference between the two portions may be less than two. Two factors are apparently of particular importance in determining the character of this relative distribution. First in import is the amount of primary contamination of the milk from the time it is drawn from the cow until it is bottled. If comparatively few bacteria gain access to the fluid the rising cream will incorporate a much higher percentage of them than when the primary contamination has amounted to several hundred thousand bacteria per c.c. A second factor which influences this relative bacterial content of cream and skim milk is the temperature at which the fluid is kept.

To determine the influence of this temperature factor a number of bottles of certified milk of high quality were placed at ice-box temperature (12° C.), at room temperature (18° to 23° C.), and at 30° C. At intervals samples from the cream and skim milk were withdrawn and plated. In Table 7 are detailed the average counts for five bottles at ice-box temperature. During the first 24 hours, the bacteria showed a tendency to decrease in number rather than multiply, but after 48 hours the rate of increase in the cream was somewhat greater than in the skim milk. Subsequently, to the time of souring, the rapidity of increase in the skim milk is only slightly in advance of that for the cream.

As the temperature at which the milk is kept is raised the relative rate of bacterial multiplication in the skim milk as compared with that in the cream becomes greater and greater. The average counts with five bottles of certified milk placed at room temperature are also given in Table 7. It will be observed that the relative rate of increase in the skim milk at 24 hours is considerably in advance of that in the ice-box series, altho this point is masked to some extent by sedimenta-

tion. As the souring-point is approached the rate of multiplication in the skim milk has so far outstripped that in the cream that the former now shows the greater degree of contamination. If one further raises the temperature of the environment to 31° C., the rate of increase in the skim milk may amount, within five hours, to many times that in the cream (Table 7).

TABLE 7.

Influence of Temperature on the Relative Rate of Increase in Cream and Skim Milk.

bottles at ice-box temperature (12° c.).

Samples	Plated "At Once"	Plated after 24 Hrs.	Ratio to "At Once"	Plated after 48 Hrs.	Ratio to "At Once"	72 Hrs. Ratio to "At Once"	96 Hrs. Ratio to "At Once"	120 Hrs.* Ratio to "At Once"
1. Top cream	18,100	25,200	1.17	530,000	29.28	251	4,423	6,608
2. Middle cream	14,600	11,700	.80	86,000	6.02	265	3,327	7,285
3. Bottom cream	10,400	7,400	.71	185,900	17.87	152	3,374	9,352
4. Top skim milk	700	500	.71	5,900	8.42	178	1,974	11,794
5. Middle skim milk	600	200	.33	2,000	3.33	333	976	8,366
6. Bottom skim milk	300	600	2.00	8,200	27.33	566	27,706	75,040

bottles kept at room temperature (18°–23° c.).

Samples	Plated "At Once"	Plated after 5 Hours	Ratio to "At Once"	Plated after 24 Hours	Ratio to "At Once"	Plated after 48 Hours†	Ratio to "At Once"
1. Top cream	28,600	35,600	1.26	14,744,000	526	113,900,000	4,069
2. Middle cream	21,200	27,900	1.31	14,202,000	676	184,000,000	8,761
3. Bottom cream	17,100	19,500	1.14	14,898,000	875	254,500,000	14,973
4. Top skim milk	900	1,200	1.33	368,700	409	219,600,000	244,000
5. Middle skim milk	600	600	1.00	233,400	389	231,800,000	386,416
6. Bottom skim milk	300	1,300	4.33	5,592,000	18,640	281,000,000	939,000

bottle kept at 31° c.

Samples	Plated "At Once"	Plated after 5 Hours	Ratio of Increase
1. Top cream	590,000	14,300,000	24.2
2. Bottom cream	370,000	10,140,000	27.4
3. Top skim milk	10,000	1,280,000	128
4. Middle skim milk	15,000	1,640,000	109
5. Bottom skim milk	1,000	1,625,000	1,625

* The cream and skim milk still sweet in all but one of the samples.

† Cream sour in all the bottles, skim milk in all but one.

DECREASE FOLLOWING SUDDEN CHANGE OF TEMPERATURE.

In investigating the effect of various temperatures on the relative increase of bacteria in the cream and the skim milk, it was observed that in some samples which were transferred from the ice-box to 30° C. and kept at that temperature for several hours there resulted a very

marked decrease in bacterial count in all parts of the bottle. Of eleven bottles so treated decided bacteriolysis occurred in three, a decrease in certain layers in three, and a rapid increase in bacterial count in five. An example of this last type is detailed in Table 7. In all samples in which the bacteria multiplied rapidly when transferred from 5° C. to 30° C. the initial count in the cream and skim milk was comparatively low. As has been observed, at 30° C. the bacteria in the skim milk multiplied far more rapidly than those in the cream and this increased rate of multiplication continued when placed at 12° C. for 18 hours. A very different result is shown in Table 8. In this instance the change from the cold to the warm environment resulted in the destruction of a large percentage of the bacteria. It seems probable that this was due to the liberation of mutually poisonous metabolic products by the various types of bacteria; in other words, it was an expression of antagonism. The milk was far too old (over 24 hours) to ascribe it to a germicidal action of the fluid itself. This antagonistic action occurred alone in samples with high counts in both the cream and the skim milk.

The bacteria in the skim milk recovered from this bacteriolysis more slowly than those in the cream. After 18 hours at 12° C. there was found to be a continued decrease in the count for the lean milk, whereas the bacteria at the top of the bottle had begun to multiply. Parallel litmus lactose agar plates exhibited a like primary decrease in the lactic acid bacilli. However, after exposure to the lower temperature they were found to have recovered more quickly than the bacteria thriving best in the more acid plain agar medium, and in fact from a comparison of the figures in the fourth column of Table 8 it is evident that they now constituted the dominant and practically exclusive type of bacteria in the milk. These dominant microorganisms were largely streptococci. If the bottle of milk is placed again at 30° C. no second bacteriolysis occurs.

An attempt was made to reproduce this antagonism experimentally by inoculating a sample of partially sterilized milk with a definite dosage of seven species of common milk bacteria, and submitting the mixture to these temperature changes. Altho there was some decrease in the count for the skim milk, the results were nothing like that detailed in Table 8. It is possible that this phenomenon under

natural conditions is dependent upon the development in milk of a peculiar and incompatible bacterial flora, which is not readily imitated experimentally.

TABLE 8.

MARKED BACTERIOLYSIS SOMETIMES OCCURRING IN BOTTLED MILK ON TRANSFERENCE FROM 5° C. to 30° C.

Samples	Plated "At Once"	Plated after 5 Hours at 30° C.	Ratio to "At Once"	Plated after 18 Hours at 12° C.	Ratio to "At Once"	Plated after 42 Hours at 12° C.	Ratio to "At Once"
Total count:							
1. Top cream..........	6,240,000	130,000	.0208	1,690,000	.27	15,000,000	2.40
2. Middle cream........	2,285,000	45,000	.019	1,105,000	.48	28,000,000	12.25
3. Bottom cream	2.100,000	10,000	.0048	910,000	.44	21,320,000	10.25
4. Top skim milk......	2,304,000	4,000	.0017	4,000	.0017	390,000	.16
5. Midde skim milk	2,145,000	24,000	.011	14,000	.0065	1,170,000	.54
6. Bottom skim milk ...	900,000	220,000	.244	19,000	.021	5,200,000	5.77
Lactic acid bacteria:							
1. As above............	487,000	25,000	.051	1,170,000	2.40	....	.....
2. As above............	266,000	20,000	.075	1,300,000	4.50	....	.....
3. As above............	140,000	9,000	.065	620,000	4.28	....	.....
4. As above............	70 000	......	.....	5,000	.07	390,000	5.57
5. As above............	90,000	1,000	.011	7,000	.07	780,000	8.66
6. As above............	26,000	......	.....	18,000	.69	1,160,000	44.5

The fact that the bacterial count in certain samples of rather grossly contaminated milk is materially reduced by an abrupt change in the temperature has apparently not been noted before. The point seems to be of some importance in the routine bacteriological examination of milk, for thereby a specimen of milk, which was primarily badly contaminated, might through the exercise of this antagonism simulate one which was obtained under proper cleanly conditions. During hot weather milk bottles in the interim between the filling at the dairy and the delivery to the customer are without doubt not infrequently subjected to as great changes in temperature as was the case in these experiments. Too few tests of this nature, however, were completed to give any idea of how often the bacterial conditions essential for this bacteriolysis obtain in milk.

SEDIMENTATION.

Contrary to what seems to be the general opinion, the sediment portion of the ordinary fresh bottle of milk embodies on the average fewer bacteria than any other portion of the fluid. This was found to be the case without exception in the certified brand of milk and frequently in the more grossly contaminated specimens. At the maximum the count was only slightly higher than the middle skim

milk and far below that of the cream. Sedimentation is evidently somewhat dependent on the rate at which the bacteria multiply in the skim milk as well as possibly on the temperature of the fluid. At low temperatures (Table 8) the increase in the sediment portion of the certified milk was not markedly greater than in the other parts of the skim milk until it had stood about 96 hours, whereas at room temperature the sediment at the end of 24 hours contained 25 times as many bacteria as the middle skim milk.

CONCLUSIONS.

1. The upper two ounces of the cream of fresh bottled milk of fair quality contain on the average 50 to 100 per cent more bacteria than an equal amount of the lower cream. In older and more grossly contaminated milk the lower cream may embody as many as or even more bacteria than the upper layers.

2. By removing these two top ounces from a milk bottle and using the remaining top milk (eight ounces) for infant feeding, as Hess has suggested, there generally results a reduction of from 30 to 50 per cent in the bacterial count.

3. The dominant controlling factor in the primary disposition of bacteria in a milk bottle is the upward "rafting" activity of the fat globules. A higher percentage of bacteria are brought to the surface layers in a milk rich in cream than one poor in that substance.

4. At ice-box temperature the rate of increase of bacteria in the cream and that in the skim milk are practically identical. As the temperature is elevated the rate of multiplication in the skim milk outstrips that of the cream until at 30° C. it may be many times as rapid.

5. In certain samples of rather highly contaminated milk the abrupt change in the temperature of the environment from 5° C. to 30° C. caused a striking bacteriolysis in both the cream and the skim milk. This was probably an expression of bacterial antagonism.

6. The sediment portion of the average bottle of fresh milk contains frequently fewer bacteria than any other region of the fluid. A marked excess of bacteria in the sediment indicates that the milk is old or that it has been kept in a warm place.

THE VALUE OF OPSONIN DETERMINATIONS IN THE DISCOVERY OF TYPHOID CARRIERS.*

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The comparatively recent discovery of so-called bacillus carriers (typhoid and paratyphoid) has lent additional interest to the question of immunity toward typhoid and paratyphoid bacilli, for it is evident that there must be some modification of the bacillus itself or of the defensive properties of the carrier's tissues to make possible the prolonged harboring of these pathogenic organisms without the occurrence of a general infection. The literature on typhoid and paratyphoid carriers is already quite extensive and I shall refer to it only as it bears on this point of the carrier's immunity to his own bacillus.

It is natural that observations on the presence of agglutinins in the carrier's serum should be far more numerous than those on the other antibodies. As a usual thing in the literature we find that the blood of the carrier has been tested for agglutination of the typhoid bacillus, either the carrier's own strain or a stock culture, and in the majority of instances agglutination has occurred tho seldom in high dilution. Förster and Kayser[1] state that the serum of chronic carriers usually contains agglutinin for the corresponding bacillus. Lentz[2] obtained agglutination in 10 of his 11 carriers in dilution of 1:50, more rarely 1:100.

That the persistence of agglutinin for long periods after recovery is probably due to the persistence of the bacilli in the body is held by Kutcher[3] and he would also explain the occasional occurrence of agglutinin for the typhoid bacillus in cases of icterus, meningitis, sepsis, etc., by assuming that such persons were in fact typhoid carriers. Steinberg,[4] who found that 15 out of 22 icteric patients agglutinated typhoid bacilli in dilution of at least 1:40, attributed these results to group agglutination caused by an infection with colon bacilli or with one of the proteus group, but Quènu[5] believes that the explanation lies in a primary infection of the gall-bladder with typhoid bacilli and F. Ehrlich[6] has reported two cases of such primary infection under the name of "biliary typhoid."[7]

* Received for publication February 1, 1910.

[1] *Münch. med. Wchnschr.*, 1905, 52, p. 1471.

[2] *Klin. Jahrb.*, 1905, 14, p. 475.

[3] Kolle und Wassermann, *Handbuch*, Ergbd. 1, 1907, pp. 260, 655.

[4] *Münch. med. Wchnschr.*, 1904, 51, p. 469.

[5] *Rev. de Chirurg.*, 1908, 38, p. 571.

[6] *Deut. med. Wchnschr.*, 1906, 32, p. 1704.

[7] A case of paratyphoid infection of this sort came under my observation in the person of a young Greek, with jaundice, fever, clay stools, etc. The patient had never had typhoid fever and this was the first attack of the present character. His index to paratyphoid bacillus B, was 0.3, 1.2, 4.1, and 5.0 the examination extending over a period of nine days. Paratyphoid bacillus B was isolated from his stools and agglutinated by his serum in dilution of 1:80.

On the other hand it has often been found that the typhoid bacillus may be present in large numbers in the body of a carrier and yet the serum show no agglutinating power above that which may be found in normal serum. Dörr[1] claims, on the basis of animal experiments, that if typhoid bacilli are present in the intestinal tract and gall-bladder only, the agglutinating power of the serum will not be above 1:22 and that a higher agglutinating power is found only when the bacilli have passed into the blood stream. Even this, however, may occur without causing any production of agglutinins, as was shown in Busse's four remarkable cases. Busse[2] isolated typhoid bacilli in pure culture from the blood of four non-typhoid patients but found that the serum of all four was without agglutinating power. His theory was that these persons were undetected typhoid carriers and that ulcerative or desquamative lesions in the intestinal tract had allowed the typhoid bacilli to pass into the blood, where their presence seemed to cause no changes. Three of the cases were in the last stages of tuberculosis, with intestinal ulcers, the other was a pneumonia case who recovered.

On the whole, then, agglutinins for typhoid bacilli are not always to be found in the blood of typhoid or paratyphoid carriers. Kamm[3] and Gaetgens[4] consider this test of very little diagnostic value for the detection of bacillus carriers.

When it comes to the question of an increased bactericidal power in the blood of carriers there are but few recorded observations and those we have are not very conclusive. A slight increase of bactericidal power was noted in Ledingham's[5] cases. Kutcher[6] also reports a high bactericidal power in the serum of one carrier whom he examined.

There is one published observation on complement deviation. Schöne[7] found complement-deviating substances in the blood of three typhoid carriers and claims that this reaction can be obtained at least as constantly as agglutination. His three carriers gave the following, only partly satisfactory, results:

		Aggl. 1:50	Complement Deviation
Case 1.	Typhoid fever 10 years ago	−	+
Case 2.	In contact with typhoid patient 2 years ago	−	−
Case 3.	Typhoid fever 12 weeks ago	+	++

The failure on the part of the great majority of observers to find any decided increase in protective substances in the serum of typhoid and paratyphoid carriers has led to a general adoption of the often quoted theory of Wassermann and Citron[8] which attributes the immunity of carriers to a local biologic change in the tissue cells as a result of prolonged contact with the bacteria in question. As numerous observers have shown the seat of infection in typhoid and paratyphoid carriers to be the biliary tract it is the lining cells of these structures which are responsible for the defense of the body. This local cellular resistance to bacteria, which may persist for a lifetime, is not due to the continued production of antibodies, for none can be demonstrated in the blood serum. It is rather an obscure biologic change in the cells, rendering them

[1] *Centralbl. f. Bakt.*, Abt. 1, Orig., 1905, 39, p. 624.

[2] *Münch. med. Wchnschr.*, 1908, 55, p. 1113.

[3] *Ibid.*, 1909, 56, p. 1011.

[4] *Deut. med. Wchnschr.*, 1909, 35, p. 1337.

[5] *Brit. Med. Jour.*, 1908, 2, p. 1173.

[6] Kolle und Wassermann, *Handbuch*, Ergbd. 1, 1907, p. 260.

[7] *Münch. med. Wchnschr.*, 1908, 55, p. 1065.

[8] *Deut. med. Wchnschr.*, 1905, 31, p. 573.

insusceptible to the attacks of the invading germs. To this acquired cellular immunity of Wassermann and Citron, Lübke[1] would add an acquired immunity on the part of the carrier's own strain, "eine erworbene Serumfestigkeit." He found that in experimentally injected animals the bacilli would persist longest in those very tissues (spleen, marrow) the extracts from which were richest in antibodies. Therefore it is impossible to gauge the resistance of an organism by the bactericidal power of the body fluids or cells, for the presence of these antibodies calls forth the production of corresponding defensive substances in the body of the bacillus. Thus in the case of bacillus carriers there is established a sort of armed truce between the antibodies of the carrier and the bacterium.

It is only in the very recent literature that we find any mention of opsonin in the blood of typhoid and paratyphoid carriers. Ledingham[2] in 1908 examined the serum of two typhoid carriers with regard to their opsonic index, using inactivated and complemented serum, and found the index very high both to the carrier's own strain and to that of the other carrier. A few weeks ago Gaetgens[3] published the results of his study of the opsonic index in 12 typhoid convalescents, who had not become carriers, and in 16 chronic typhoid carriers and 2 chronic paratyphoid carriers. He found that typhoid convalescents who did not become carriers exhibited an opsonic index above normal only for a short period, three or four months at the outside, while typhoid carriers had a persistently high index, irrespective of the lapse of time since recovery from typhoid fever or even in the absence of any history of typhoid fever. As all but one of these carriers had an index above 1.4, the average being 2.8 (Gaetgens uses unheated serum and his counts are therefore not as high as Ledingham's), and as 25 per cent had failed to agglutinate in dilutions higher than 1:50, Gaetgens considers the opsonic index of more value in the detection of carriers than the agglutination test. His two paratyphoid carriers, on the other hand, gave only normal indices, a result which he thinks may be due to the fact that the laboratory strain used had lost its virulence, but he believes that possibly this may prove to be a distinction between typhoid and paratyphoid carriers.

One cannot help suspecting that the case recently reported by Aaser[4] may have been a typhoid carrier. The man had had typhoid fever in 1898, his serum had lost all agglutinative power six months after his recovery, but when his opsonic index to the typhoid bacillus was taken in 1907, it was found to be very high, 6.0 and 7.0.

It is interesting to note the increasing number of observations in the literature which tend to show that the immunity on the part of bacillus carriers is, after all, only partial in most cases and liable to complete breakdown. The presence in the gall bladder and passages of typhoid or paratyphoid bacilli leads frequently to a chronic cholecystitis with or without the formation of stones, and recurring attacks of fever and jaundice are not rare in the history of carriers. Lentz[5] says that there is a direct relation between slow recovery with lasting ill-health and a persistence of the germs in the body. Dean's[6] and Schiller's[7] cases apparently confirm this statement. In

[1] *Münch. med. Wchnschr.*, 1909, 56, p. 57.

[2] *Brit. Med. Jour.*, 1908, 2, p. 1173.

[3] *Deut. med. Wchnschr.*, 1909, 35, p. 1337.

[4] *Jahresb. f. Immunitätsforschung*, 1908, 3, p. 86.

[5] *Klin. Jahrb.*, 1905, 14, p. 475.

[6] *Brit. Med. Jour.*, 1907, 50, p. 562.

[7] *Wien. med. Wchnschr.*, 1908, p. 12.

other cases the bacillus lodges in different parts of the body, setting up a suppurative process, many months after recovery from the original sickness or even without any history of typhoid fever. Bauer[1] has collected eight instances of suppurative chondritis due to the typhoid bacillus, occurring from two months to two years after recovery from typhoid fever. Kamm[2] reported two similar cases, one of whom had no history of typhoid fever.

Two striking instances of complete loss of immunity in old typhoid carriers have been described by Levy and Kayser[3] and by Grimme.[4] In both of them there was a history of typhoid fever, followed by persistence of the bacilli in the biliary tract, a chronic cholecystitis with stones (in both cases the stones yielded cultures of typhoid bacilli), and finally a re-autoinfection from the gall bladder, causing fatal typhoid bacteriemia. Another, but not fatal case, of the same general infection occurring in a carrier with cholecystitis is reported by F. Ehrlich.[5]

The following study was undertaken with the hope of adding to the small number of observations now available on the antibodies in the serum of typhoid and paratyphoid carriers, especially the opsonin, but in the course of the work other questions arose which will also be discussed briefly.

As no known typhoid carriers were available, I chose for my search a class of patients who would supposedly yield a larger proportion of carriers than any other, namely, persons with chronic cholecystitis. It is unnecessary to review the extensive literature which has shown that chronic inflammatory lesions of the biliary tract are in many instances caused by typhoid or paratyphoid bacilli. More recently the reports on typhoid carriers have laid stress on the frequent presence of gall-stones in the carriers and upon the fact that the proportion of multiparous women among bacillus carriers is similar to the proportion of multiparous women suffering from gall-stones[6] (Förster and Kayser;[7] Muller[8]).

Of the 24 cases selected, all but one were patients with various lesions of the gall tract. Twenty were middle-aged married women, one was an unmarried women of 50 years, and one a man of 28 years. The twenty-fourth was a woman in normal health who had a history

[1] Inaugural Diss., Rostock, 1894.

[2] *Münch. med. Wchnschr.*, 1909, 56, p. 1011.

[3] *Arb. a. d. kais. Gesundh.*, 1907, 25, p. 254.

[4] *Münch. med. Wchnschr.*, 1907, 54, p. 1822.

[5] *Deut. med. Wchnschr.*, 1906, 32, p. 1704.

[6] A more conservative stand on the question of the relation between typhoid infection and cholecystitis is found in a recent article by Eug. Fraenkel, *Mitth. a. d. Grenzgebiet. d. Med. u. Chi.*, 1909, 20, p. 898.

[7] *Münch. med. Wchnschr.*, 1905, 52, p. 1471.

[8] *Am. Jour. Med. Sci.*, 1908, 126, p. 314.

of both typhoid and paratyphoid fever. Five had had typhoid fever, but only one of them as recently as four years ago. This last case traced her present trouble directly to an unusually severe attack of typhoid fever. A brief résumé of the important facts in the histories of these patients is given in Table 1 and I shall not go into details now except to state that at the time the examination was made four of the patients were suffering from an acute attack of colic, jaundice, fever, clay stools, etc., and gave a history of similar symptoms in the past. The other 20 were at the time free from acute symptoms.

The principal results of the study of these cases would come under the following heads: A. The large proportion of carriers among cases of chronic cholecystitis. B. The incompleteness and instability of the carrier's immunity to his own bacillus. C. The abnormal opsonic index to typhoid or paratyphoid bacilli in the serum of carriers. D. The close relation between typhoid and paratyphoid bacilli.

THE LARGE PROPORTION OF CARRIERS AMONG CASES OF CHRONIC CHOLECYSTITIS.

The following examinations were made in these cases:

1. The patient's stool, urine, and, in operative cases, bile or pus, were examined for the presence of typhoid or paratyphoid bacilli. Malachite green agar plates (containing malachite green 1:2,000 or 1:2,500) were spread with the material in question, allowed to stand for 24 hours at 37° C., the colonies then washed off with salt solution and spread upon plates filled with Endo and with Drigalski-Conradi medium. This malachite green agar had been found to inhibit the growth of three laboratory strains of colon bacilli, while control strains of typhoid and paratyphoid bacilli grew well upon it.

2. The patient's serum was used for agglutination tests (macroscopic) with a stock strain of typhoid bacillus, one of colon bacillus, and two strains of paratyphoid bacilli, type B and type A. The strain of type B was kindly furnished by the Bacteriological Laboratory of the University of Chicago, together with four other strains which were frequently used for comparison in cultural and agglutination tests.

3. The opsonic index of these four organisms was also estimated, the patient's serum and the control serum being heated first to 55°–60° C. for 20 minutes. As this exposure is enough to inactivate the lysin in both normal (see Klien;[1] Clark and Simonds[2]) and immune serum, the difference in the action of the thermostable opsonic substance in the two becomes much more striking than in unheated specimens. The same strain of typhoid bacilli and the same strains of paratyphoid bacilli, A and B, were used in all the tests. None of them was spontaneously phagocytable in the suspensions employed.

[1] *Bull. Johns Hopkins Hosp.*, 1907, 18, p. 245.

[2] *Jour. Infect. Dis.*, 1908, 5, p. 2.

4. In patients who had acute symptoms a bacteriological examination of the blood was made by allowing from 2 to 5 c.c of sterile blood to run into a test tube containing 5 to 10 c.c. of sterile ox bile. After 24 hours' incubation the culture was spread on Drigalski-Conradi plates.

5. Bacteriolytic experiments were made with the patient's blood in all but one of the positive cases, the method described by Neufeld and Hüne[1] being followed.

The typhoid bacillus was identified culturally by the production of typical colonies on agar plates and on Endo and Drigalski-Conradi plates, by the growth in milk, litmus whey, neutral red agar, and on potato. Agglutination tests were made with the serum of rabbits injected with a laboratory strain of typhoid bacilli and, as a final test, Castellani's absorption method[2] was used.

In identifying the paratyphoid bacilli less stress was laid on cultural characteristics than upon agglutination with sera of rabbits which had been injected with control strains of paratyphoid bacilli. Rabbits were injected with the same strains of paratyphoid A and B which were used in the agglutination and opsonic tests. These immune sera showed group agglutination for the control strains of typhoid, paratyphoid, and colon bacilli. The serum of the rabbit injected with paratyphoid A agglutinated one stock strain of paratyphoid B and agglutinated feebly three colon strains. That of rabbit paratyphoid B did not agglutinate paratyphoid A or the strains of colon bacilli, but did agglutinate two strains of typhoid bacilli almost as strongly as it did the strain of paratyphoid B used in immunization. It failed to agglutinate three stock strains of paratyphoid B.

In the course of the examination of the 24 cholecystitis cases, 12 organisms were isolated two of which proved to be typhoid bacilli, typical in all respects. Three belonged to the colon group but were not typical, in that they produced but very little gas in glucose media, grew well upon the malachite green plates, and formed pink colonies on Endo plates instead of the deep red ones with metallic luster which are typical of colon bacilli. They also agglutinated strongly with the serum of the rabbit immunized against paratyphoid A. The remaining 7 organisms were apparently paratyphoid bacilli, but it would be difficult to classify all of them on the ground of cultural characteristics alone. Three corresponded to the usual description

[1] *Arb. a. d. kais. Gesundh.*, 1907, 25, p. 164.

[2] *Ztschr. f. Hyg.*, 1902, 40, p. 1.

of type B except that they formed a typhoid-like growth on potato. They also agglutinated with type B immune serum and I have therefore classified them under that head. One agglutinated with the same serum and formed a colon-like growth on potato—belonging also to type B. The fifth belonged culturally to this type, but agglutinated with serum type A. The sixth agglutinated only with the patient's serum and culturally resembled type A, while the seventh was apparently a typical member of type A both culturally and by serum tests. Naturally, all the strains which resembled the colon bacillus culturally and which failed to agglutinate with immune rabbit serum or with the patient's serum were rejected.

Of the 24 cases examined only those were pronounced to be carriers who fulfilled the following requirements:

1. The presence in the patient's body of a more or less motile, gram-negative bacillus, agglutinated by the serum of rabbits immunized against typhoid or paratyphoid bacilli, or by the patient's serum.

2. The presence in the patient's serum of an abnormal opsonic index for stock strains of the typhoid bacillus or paratyphoid bacillus B or A, and of agglutinin or opsonin for the patient's own bacillus.

Seven of the 24 patients presented these features, five being paratyphoid carriers, one a typhoid carrier, and one harboring both typhoid and paratyphoid bacilli. This represents a proportion of 29 in a hundred, and corresponds to the results obtained by Blumenthal,[1] who examined 17 cases of cholecystitis and found that four harbored typhoid bacilli and one paratyphoid type A, making a proportion of 29 in a hundred.

Three of my seven positive cases had a history of typhoid fever, two of the seventeen negative cases had such a history. It is interesting to note that neither typhoid carrier had ever, so far as could be discovered, been the cause of infection in others. One was a woman of wealth who did not handle the food of the household, the other prepared the food for her own family but had very cleanly habits.

IMMUNITY OF CARRIER TO HIS OWN BACILLUS.

In only one of the seven carriers was there complete immunity to the infecting agent, altho the histories of these cases showed that

[1] *Mediz. Klinik*, 1905.

the bacilli had been harbored for periods running from two months to eight years. Three gave histories of repeated attacks of biliary colic, and at the time of examination were recovering from operative removal of gall-stones. One was waiting operation and during this time had two attacks of fever and jaundice. Another was recovering from an operation for abscess of the liver and the sixth was suffering from his second attack of acute catarrhal jaundice. The one case who was apparently quite normal gave a history of typhoid fever 12 years ago and of paratyphoid fever (clinically diagnosed) four months before. She was one of the paratyphoid carriers.

Reference has been made to the three cases of typhoid bacteriemia reported by Levy and Kayser, by Grimm, and by Fr. Ehrlich. One similar case is included in my series, that of a paratyphoid carrier who developed a general bacteriemia following an operation for the removal of gall-stones and ending in recovery. This woman, Case No. 5, was a patient of Dr. C. H. McKenna[1] who has already published a clinical report of the case.

The important features from my point of view were the following: a history of an attack of cholecystitis dating back more than two years; a second attack during which an operation was performed, this being apparently the occasion for the escape of paratyphoid bacilli type A into the blood stream.[2] The patient's immunity to her bacillus was evidently lowered at the time of the attack and the operation caused a still greater loss of resistance. As will be seen later, it was possible in this case to show a loss of opsonin at the time of the bacteriemia and a very marked increase as convalescence set in.

ON THE PRESENCE OF ANTIBODIES IN THE SERUM OF TYPHOID AND PARATYPHOID CARRIERS.

Agglutinins.—Gaetgens rejects all sera which agglutinate in dilution not higher than 1:50. Lentz accepts 1:50 as positive. Dörr found that the serum of rabbits with experimental typhoid cholecystitis agglutinated in dilution no higher than 1:22. Bieberstein, reviewing the literature on agglutination in typhoid fever, found a decided difference of opinion as to what dilution of serum should

[1] *Jour. Am. Med. Assoc.*, 1909, 1, p. 239.

[2] This case is similar in all essentials to that reported by Libman, *Jour Med. Res.*, 1902, 3, p. 168..

be regarded as the limit beyond which a positive diagnosis should be made, the figures running from 1:20 to 1:50. He puts it at about 1:30, as he has found only 2 per cent of normal sera agglutinating at this point. Three of my five paratyphoid carriers agglutinated a stock strain or their own strain of paratyphoid or both in dilution of 1:50 or higher. Both typhoid carriers agglutinated typhoid bacilli, stock strain and homologous strain, in dilutions higher than 1:50, so that five out of seven carriers, or 71 per cent, gave positive results with the agglutination test. The case of proved mixt infection, the typhoid and paratyphoid carrier, agglutinated both organisms in dilutions of 1:80 and 1:100. Group agglutination was found in Case 4 whose serum agglutinated typhoid as well as paratyphoid bacilli without any other evidence of mixt infection, i.e., without the discovery of typhoid bacilli in the patient's body and without a high opsonic index to the stock strain which was agglutinated.

Opsonins.—Table 1 gives the agglutinations and opsonic indices in the eight carriers. This table shows that all seven carriers had an abnormal opsonic index for their own strain or for some stock strain of typhoid or paratyphoid bacilli. In three instances, Cases 4, 5, and 7, the paratyphoid bacilli isolated from the pus, blood, and urine respectively were at first non-phagocytable, but the patient's indices were found to be abnormal to stock strains of paratyphoid bacilli and later on to their own strains. The opsonic index therefore proved positive in 100 per cent of all carriers as against the 71 per cent of positive agglutination tests. These results correspond with those of Gaetgens quoted above.

Among the non-carriers were two, both former typhoid cases, who agglutinated typhoid bacilli in low dilution, 1:30 and 1:40. Another agglutinated paratyphoid A, 1:30. No decidedly high opsonic index was found among the non-carriers altho occasionally an index from 2.0 to 2.7 would be found in a serum which was usually normal. This was not regarded as significant in the absence of any other positive findings, for it could easily be explained on the ground of group opsonic action in a case of infection by one of the colon group. The two cases of mixt infection, Cases 1 and 2, had high indices for typhoid bacilli and for their own strains of colon bacilli, atypical, and Case 1 for paratyphoid B as well. Here opsonin and agglutinin corresponded.

Cases 4, 5, 6, and 7 had an abnormal index only for one organism, namely that corresponding to the strain later isolated. No group opsonic action could be shown in these cases. On the other hand, Case 3 had a high index to typhoid as well as to paratyphoid bacilli, altho she could not be shown to harbor any but the last-named organism. The same thing was true of Case 8, whom I have inserted with the others in the table because, tho not a carrier, the findings in her case corresponded so closely with those in Case 3. She too had a

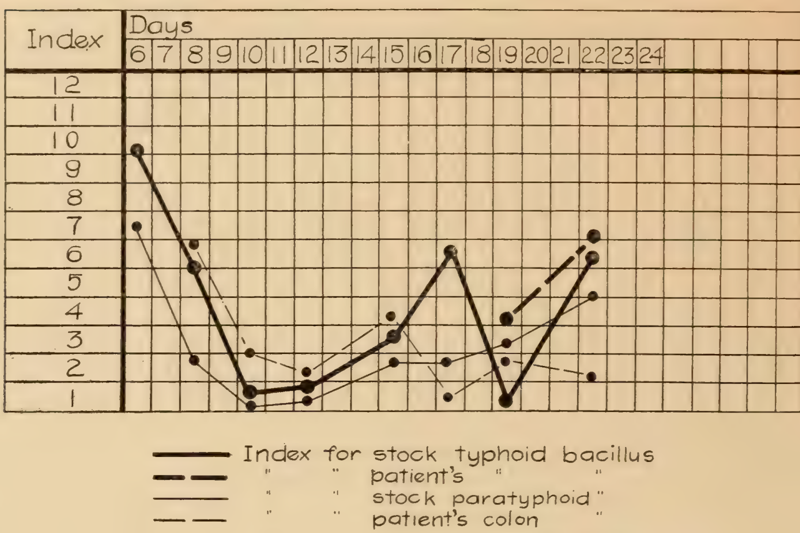

CHART 1.—Opsonin index of Case 2. Acute typhoid cholecystitis.

high index to typhoid bacilli as well as to her own strain of paratyphoid type B, but no typhoid bacilli were ever found in the course of repeated examinations of her stools. These anomalous opsonic indices will be discussed later in their bearing upon the question of cross opsonins, but I mention them here to show that while the opsonic test proved positive in all my carriers, it did not always serve to indicate whether the case was a typhoid or a paratyphoid carrier or both.

A study of the charts made from cases with acute symptoms shows that the amount of opsonin present fluctuated according to the increase or subsidence of the disturbance. Case 5 had a low index shortly after her operation, at the time when the blood contained

TABLE 1.

AGGLUTINATION AND OPSONIC INDEX OF CARRIERS.

No.	Sex and Age	Typhoid Fever	Hospital	Condition	Bacillus Found in	Bacilli Isolated	Agglutination: Typhoid Bacillus	Agglutination: Paratyphoid Bacillus A	Agglutination: Paratyphoid Bacillus B	Agglutination: Colon Bacillus	Opsonic Index: Typhoid Bacillus	Opsonic Index: Paratyphoid Bacillus A	Opsonic Index: Paratyphoid Bacillus B	Opsonic Index: Colon Bacillus
1. Typhoid and paratyphoid carrier	Married woman, 45 yrs.	4 years before	Presbyterian Hospital Dr. Bevan	Cholecystitis 4 years Operation: adhesions, constriction of cystic duct	Urine Bile Stool	Colon bacillus (atypical) Paratyphoid bacillus, type B Typhoid bacillus	+1:200 S. and P.*		+1:300 P.	+1:150 P.	6.0:16.0 S. and P.		3.0:8.0 S. and P.	2.2:4.6 P.
2. Typhoid carrier	Married woman, 50 yrs.	No	Presbyterian Hospital Dr. Billings and Dr. Bevan	Cholecystitis 4 years Acute attack Operation: Stones	Urine Bile Stool	Colon bacillus (atypical) Typhoid bacillus	+1:100 S. and P.			+1:80 P.	1.1:9.8 S. and P.			1.1:6 8 P.
3. Paratyphoid carrier	Unmarried woman, 34 yrs.	12 years before. Paratyphoid fever 4 months before	West Side Hospital Dr. Van Hoosen	Normal	Stool	Paratyphoid bacillus, type B	+1:30 S.		+1:30 P.	...	5.2:21.0 S.		3 3:15.0 P. and S.	
4. Paratyphoid carrier	Married woman, 46 yrs.	No	Presbyterian Hospital Dr. Herrick and Dr. Bevan	Abscess of liver Operation	Pus from abscess	Paratyphoid bacillus, type B	+1:50 S.		+1:150 S. and P.		1.9:2.7		0.53:12.5 P. and S.	
5. Paratyphoid carrier	Unmarried woman, 40 yrs.	No	St. Joseph's Hospital Dr. McKenna	Second acute attack First attack 2½ years ago Operation: Stones Bacteriemia	Blood	Paratyphoid bacillus, type A		+1:300 P. and S.				0 6:31 0 P. and S.		
6. Paratyphoid carrier	Man, 28 yrs.	5 years before	Presbyterian Hospital Dr. Billings	Second acute attack First attack 2 months ago	Stool	Paratyphoid bacillus, type B			+1:80 S.				1.4:10.0 P. and S.	
7. Paratyphoid carrier	Married woman, 32 yrs.	No	Cook County Hospital	Cholecystitis 8 years Operation: Stones	Urine	Culturally like paratyphoid bacillus, type B. Agglutinated with type A serum						3.5:6 0 S.		
8. Not shown to be a carrier	Unmarried woman, 33 yrs.	Had nursed typhoid cases	Presbyterian Hospital Dr. Herrick	Paratyphoid fever	Urine Stool	Paratyphoid bacillus, type B Colon bacillus (atypical)	+1:40 S.		+1:200 P. and S.	+1:40 S.	4.0:22.0 S.		1.0:14 0 P. and S.	2.0:10.0 P.

* S. = stock: P. = patient's strain.

paratyphoid bacilli. It rose gradually and reached 31.0 during convalescence, then sank to 5.5, and after complete recovery, seven months after her operation, it stood at 11.0. Case 4 had an index of 0.53 at the time when her temperature was high and there was a profuse discharge from the liver abscess. Before leaving the hospital

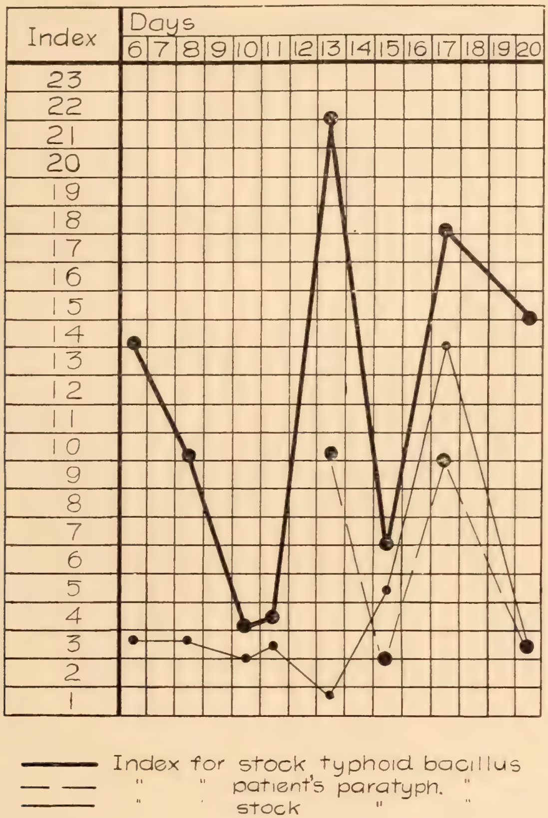

CHART 2.—Opsonin index of Case 8. Paratyphoid fever.

her index had risen to 12.5. The charts of Cases 2 and 8, which are reproduced, show how the index varied with the course of the disease. The chronic cases on the other hand, without acute symtoms, had a fairly steady, high index, rising not much over 8.0, seldom falling below 3.5 and never below normal. When double infection existed, and in those cases in which, without demonstrable double

infection, high indices for two different organisms were found, the indices to the different organisms were strikingly similar (Charts 1 and 2).

It was not possible in most of these cases to show a diminution in the carrier's opsonin toward her own strain as compared to stock strains nor greater resistance to phagocytosis on the part of the carrier's strain as compared to stock strains. Three times only, in Cases 4, 5, and 7, the strain of paratyphoid isolated from pus, blood, and urine respectively exhibited a marked resistance to phagocytosis in the presence both of normal and of the patient's serum. This characteristic was, however, soon lost (except by the bacillus of Case 7) and it may be that the stage of resistance in the other strains had already passed when the organisms were tested, for usually several days of cultivation on artificial media had elapsed before it was possible to decide on the character of the strain and make the opsonic tests.

Bacteriolysin.—Experiments to determine the bactericidal power of the carrier's serum for laboratory strains, and for the individual strain isolated, were made in Cases 1, 2, 3, 4, 5, and 8. Case 7 was tested only with laboratory strains. The carrier's serum and the control normal serum were inactivated by heat and complemented with normal rabbit serum, following the method advised by Neufeld and Hüne.[1] In no case was there any striking or constant difference between the carrier's serum and the normal serum, not even in the case of the patient with paratyphoid bacteriemia. Nor was it possible to show an increase in resistance on the part of the carrier's strain as compared with the control strain.

THE CLOSE RELATION BETWEEN TYPHOID AND PARATYPHOID BACILLI.

There are a large number of articles in the literature on the group agglutination of the different members of the typhoid-colon group of bacilli, all tending to show that the members are more or less closely related to each other. Agglutination of paratyphoid bacilli by the serum of typhoid patients and also the converse phenomenon have been reported by Schottmüller, Drigalski, Schultz, Kayser, Seiffert, Lentz, Kutcher, Grünberg and Rolly, and Korte and Steinberg.[2]

[1] *Arb. a. d. kais. Gesundh.*, 1907, 25, p. 164.

[2] A full review of the literature is to be found in Kutcher's article in Kolle and Wasserman's *Handbuch*, Ergbd. 1, 1907, p. 188.

Some observers have found that the serum in question agglutinated the allied bacillus more quickly than the homologous bacillus (Lentz) or in higher dilution. Grünberg and Rolly found that 70 per cent of typhoid patients agglutinated paratyphoid bacilli; 35 per cent of them in higher dilution than typhoid bacilli; 15 per cent agglutinated colon bacilli also. Drigalski had 26 among 275 typhoid patients whose serum agglutinated paratyphoid bacilli more strongly than typhoid. Bieberstein found that in 5 of his 18 typhoid patients the serum agglutinated colon bacilli in higher dilution than typhoid bacilli.

As for the opsonins, according to the few reports that have appeared, the same group action exists as in the case of agglutinins. Schottmüller and Much[1] found the index of typhoid patients high for paratyphoid as well as for typhoid bacilli, and Clark and Simonds[2] found that the index might be even higher for the former than for the latter.

Such results are explained in one of two ways: either on the ground of a mixt infection resulting in the formation of different agglutinins, or on the ground of related agglutinins, the bacilli of this large group being supposed to cause the formation of antibodies which affect to a certain extent the other members of the group. Gaetgens lays great stress on the frequency of mixt infection, especially in typhoid fever, and describes three ways in which this may take place: first, an actual double infection at the outset; second, the entrance of paratyphoid bacilli as saprophytes during the course of typhoid fever; and third, the late appearance and gradual increase of paratyphoid bacilli during convalescence from typhoid fever. That those cases which come under the first and third heads are really mixt infections can be shown by the absorption test. In those cases where paratyphoid bacilli appear during convalescence from typhoid fever, a prolonged observation shows that as the typhoid bacilli disappear the paratyphoid increase, suggesting either that the former are being displaced by an antagonistic organism or that the typhoid bacillus is undergoing a modification. Gaetgens discusses the possibility that paratyphoid bacilli may represent a modified form of typhoid bacilli but rejects the theory as unproven. Still

[1] *Münch. med. Wchnschr.*, 1908, 55, p. 435.
[2] *Ibid.*, p. 496.

there are facts which point to a specially close relationship between the two. For instance, he found in his typhoid convalescents that altho the paratyphoid bacilli appeared in all cases, usually about the sixth week, their appearance was not accompanied by any clinical symptoms, a fact which seemed to indicate that the antibodies already formed against the typhoid bacillus were active against paratyphoid also. He then cites instances of temporary paratyphoid carriers who were at the time in contact with cases of typhoid fever, not paratyphoid. Two chronic paratyphoid carriers with cholelithiasis were carefully examined for the presence of typhoid bacilli but with negative results, yet there was a strong suspicion that they were responsible for the cases of typhoid fever in their environment. Another carrier had a history of typhoid fever at six years of age. At the time of examination her two children had typhoid fever, but her stools showed only paratyphoid bacilli.

Kayser's observations are quoted by Gaetgens[1] in confirmation of his own. Kayser emphasizes especially the importance of paratyphoid bacillus type A as a factor in the mixt infection in typhoid fever. This bacillus may be agglutinated earlier and more completely than the typhoid bacillus in cases of clinical typhoid fever. It may even be grown in pure culture in such cases and agglutination tests prove it to be the pathogenic agent (Blumenthal[2]). Kayser does not consider the agglutinins for typhoid bacilli and paratyphoid bacillus A really specific. He, like Gaetgens, has noted the appearance of paratyphoid bacilli in convalescence from typhoid fever as has also Seiffert.[3]

Among my seven carriers there were three who seemed to illustrate this close relationship between the members of the typhoid-colon group. Case 1 had a history of undoubted typhoid fever four years previously, during which attack typhoid bacilli were isolated from her blood. Her cholecystitis was a sequence of this illness. At the time of my first examination, during the first week after her operation, bile, stools, and urine contained large numbers of paratyphoid bacilli but a most careful search failed to reveal any colonies of typhoid

[1] *Deut. med. Wchnschr.*, 1904, 30, p. 1803.

[2] *Münch. med. Wchnschr.*, 1904, 51, p. 1641.

[3] *Ztschr. f. Hyg.*, 1909, 63, p. 272. See also the epidemic of paratyphoid (B) fever following infection of food by a typhoid carrier described by Fornet, *Arb. a. d. kais. Gesundh.*, 1907, 25, p. 247.

bacillus. Yet at this time the patient's serum gave the following agglutinations: Typhoid bacillus 1:100, paratyphoid bacillus B 1:200. Her opsonic index for the former was from 6.0 to 16.0; for the latter 3 to 8. This proof of the presence of antibodies for the typhoid bacillus induced me to continue my search for this organism, but it was not until 17 weeks later, when the woman was in perfect health, that the plates from her stools yielded, among numerous colonies of paratyphoid bacillus B, a very few colonies of typhoid bacillus. A subsequent examination, three months later, gave only paratyphoid bacilli. This woman's serum at the time of the last examination in April (the date of the operation was November 11) agglutinated her own strain of typhoid bacillus and a laboratory strain in dilution of 1:40, and had an opsonic index of 10 to these strains. At the same time the serum agglutinated her own paratyphoid bacillus in dilution of 1:40 and the laboratory strain in 1:100. Her index was 5.0 to the former, 3.0 to the latter. Castellani's absorption test showed that after the removal of the typhoid agglutinins from the serum, the agglutinins for paratyphoid bacilli were still active. In this case, then, we have apparently a confirmation of the statement that a paratyphoid infection may follow and displace a typhoid infection. There can be no doubt that the cholecystitis was due in the first instance to a typhoid inflammation of the gall-bladder, but at the end of four years' time those bacilli had almost disappeared, altho the serum still was rich in antibodies. Their place had been taken by paratyphoid bacilli which in this case could not be regarded as simply saprophytes because of the specific agglutinin and opsonin for these bacilli in the woman's serum.

Case 2 was in some respects similar, but without a history of typhoid fever. She entered the hospital with the history of frequent attacks of colic, chill, fever, and jaundice. Her blood agglutinated typhoid bacilli in dilution of 1:70; and paratyphoid in dilution of 1:40 only. Her index to the former was 9.8 and to the latter 7.5. No typhoid bacilli could be found in urine or stools at that time.[1] An acute attack of fever and jaundice was followed by a fall in her opsonic index for typhoid bacillus and paratyphoid B (see Chart 1) and as

[1] Most of the typhoid carriers reported in the literature excreted great numbers of these bacilli (see Scheller, *Centralbl. f. Bakt.*, Abt. 1, Orig., 1908, 46, p. 385). Klinger, however, found only very few colonies in the plates from his carriers (*Arb. a. d. kais. Gesundh.*, 1906, 24, p. 91).

she convalesced from this attack the indices rose again. A second attack had the same effect and it was at this time that a few colonies of typhoid bacilli were found in the plates from the clay-colored stools. Shortly after this the patient was operated on for the removal of gall-stones and the bile at the time of operation yielded an almost pure culture of typhoid bacillus. Her serum agglutinated her own strain of typhoid bacilli in dilution of 1:100 and her index at two observations was 3.0 and 7.0. There was no reason to suspect this woman of being a typhoid carrier aside from the discovery of antibodies for the typhoid bacillus in her serum. Not only did she deny any history of typhoid fever, but she had never nursed or even been in contact with any case of typhoid fever, and as all the early examinations of her stools were negative she would certainly have escaped detection if the high opsonic index and agglutinative power of her serum had not pointed to the presence of typhoid infection somewhere.

The two following cases were harder to interpret. Case 3 had had typhoid fever 12 years previously and an attack of what was diagnosed as paratyphoid fever 4 months before. She was in perfect health at the time of the examination and her serum did not agglutinate either typhoid or paratyphoid bacilli to any significant extent (B. typhosus 1:30) but did show an index of 5.2 to 21.0 for typhoid bacilli and of 3.3 to 15.0 for paratyphoid B. Only three specimens of stools could be obtained, from two of which a paratyphoid bacillus type B was obtained, but no typhoid colonies could be found.

Case 8 was not a carrier but was suffering from an attack of paratyphoid fever. She was a trained nurse and had been frequently in contact with typhoid patients but had never had typhoid fever. Her stools contained enormous numbers of paratyphoid bacilli which were agglutinated by her serum in dilution of 1:150 and her opsonic index ran between 1.9 and 14.0. No typhoid bacilli were ever found in the stools, yet her index to this organism was always high, between 4.0 and 22.0, and her serum agglutinated it in dilution of 1:40.

It might be said that in these last two cases we have to do with group agglutinins and group opsonins, as no mixt infection was proven, but it is at least possible that these patients were formerly typhoid carriers and that the gradual substitution of paratyphoid for typhoid

bacilli was so nearly complete as to make detection of these last impossible, though the production of antibodies still continued. If the high opsonic index in these cases and the presence of agglutinin for typhoid bacilli were to be regarded as due only to the group action of the antibodies formed in paratyphoid infection, then the same features should have been encountered in Cases 5, 6, and 7, all of whom, however, had normal indices to typhoid bacilli and no agglutinin, at the same time that they had high indices and agglutinin for paratyphoid bacilli. Case 4 lies between the two, and might be explained as an instance of group action, for while the serum agglutinated typhoid bacilli in dilution of 1:50, the opsonin for the typhoid bacillus was never very much increased. It is worth noting that there was no increase of opsonin for paratyphoid type A in the serum of carriers of type B. For instance Case 3 had an index of 12.0 to type B and of 1.2 to type A. Case 9 had an index of 5.2 to type B and a normal index to type A.

Bieberstein's work on group agglutinins for typhoid bacilli and colon bacilli was referred to above. No instance of agglutination of stock strains of colon bacilli was found in my cases, but, on the other hand, there were three cases who had both agglutinin and high opsonic index to their own strain of colon bacilli, namely Cases 1, 2, and 8. The sera of these patients agglutinated their own strains in dilutions of 1:50 to 1:150 and their opsonic indices rose to 4.6, 6.8, and 10.0 respectively. In Cases 1 and 2 Castellani's absorption test showed that the agglutinins for the homologous strains of colon bacilli were not removed by the removal of the agglutinin for typhoid bacilli, so that here we have to do with real mixt infection.[1]

SUMMARY.

Among 24 cases of chronic cholecystitis, five paratyphoid carriers were found, one typhoid carrier, and one with both organisms.

Five, or 71 per cent, agglutinated their own bacilli or stock strains

[1] I was inclined to regard these organisms as nearer to the paratyphoid group than to the colon group, for the serum of all these patients was quite normal to stock strains of colon bacilli while their opsonin and agglutinin for their own strains corresponded to their opsonin and agglutinin for paratyphoid bacillus B. Culturally the bacilli differed from typical colon bacilli in their growth on gelatin, malachite green agar, Endo plates, and in the fact that they agglutinated with the serum of rabbits immunized against paratyphoid bacillus type B, which serum failed to agglutinate stock strains of colon bacillus.

or both, in dilution of 1:50 or higher. No non-carrier agglutinated any strain in dilution as high as 1:50.

All seven had an abnormal opsonic index to their own bacilli or to some stock strain or to both. In cases with acute symptoms the index fluctuated, falling below normal at times and again rising very high, while in cases free from acute symptoms the index was persistently high, never falling to normal.

Where mixt infection by two organisms existed, agglutination and an abnormal opsonic index for both organisms were found. In one carrier, and in one case of paratyphoid fever, mixt infection was suspected because of the high opsonic index to the two organisms but it could not be proven.

The opsonic index is a very valuable aid in the discovery of bacillus carriers. No decidedly abnormal index was found in any of the non-carriers.

No proof of increased bactericidal power in the serum of carriers was found, nor any proof of increased resistance to bacteriolysis on the part of the carrier's strain. Neither was there any evidence of a greater opsonic power in the carrier's serum toward his own strain, but in three cases the carrier's strain was markedly resistant to phagocytosis.

The statement that a paratyphoid infection frequently follows or accompanies a typhoid infection was apparently confirmed by the study of these carriers.

A close relation exists between the members of the typhoid-colon group, as can be seen by group agglutinins and group opsonins and by the frequent occurrence of mixt infections.

A STUDY OF PNEUMOCOCCI FROM CASES OF INFECTIOUS ENDOCARDITIS.* †

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Certain cases of endocarditis with prolonged course reported by Schottmüller[1] and Horder[2] are similar clinically to those studied by Dr. F. Billings[3] and myself,[4] and the descriptions of the organisms isolated are very similar. Schottmüller designates his organisms as "Streptococcus mitior viridans," because they form long chains, are small in size, and produce green on blood-agar. He did not use inulin for differential tests, nor did he study the effects of animal passage. Horder names his organisms "saprophytic streptococci" on account of their lack of virulence and to distinguish them from Streptococcus pyogenes. The organisms studied by me appeared to be modified pneumococci, at least in most cases, and I now wish to present some of the results of a rather prolonged study of similar cocci from other cases of chronic infectious endocarditis.

FERMENTATIVE AND OTHER CHARACTERISTICS OF THE PNEUMOCOCCI IN ENDOCARDITIS.

Fluctuations in the fermentative powers of the cocci from endocarditis, as well as of the pneumococci isolated from pneumonia, have been observed repeatedly. Pneumococci from the sputum in pneumonia ferment inulin more strongly than those isolated from the blood, while pneumococci from cases of endocarditis ferment this carbohydrate still less and sometimes not at all. The following observations will illustrate this point: Repeated tests of the fermentative properties of the strain isolated on the many occasions in Case 362 (this Journal, p. 429) show that approximately only one-half of the colonies obtained from the blood on blood-agar plates fermented inulin. Similar results were obtained in the other cases studied.

The strain isolated July 7 from Case 408 (p. 431) fermented

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[1] *Münch. med. Wchnschr.*, 1903, 50, p. 849.

[2] *Quart. Jour. Med.*, 1909, 2, p. 289.

[3] *Arch. Int. Med.*, 1909, 4, p. 409.

[4] *Jour. Infect. Dis.*, 1909, 6, p. 245.

inulin moderately in 48 hours. Subcultures were now made on agar, in normal blood, and in the patient's blood, and transferred in each medium every second day for 30 days. At the end of this time the agar-grown culture fermented inulin in 24 hours. The subculture in blood failed entirely to produce acid, altho an abundant growth was obtained; it was then transferred to blood-agar for five days, then to sugar-free dextrose broth for 24 hours, and it now fermented inulin again, but more slowly than the agar-grown brand. Four weeks later all these strains after preservation on blood-agar fermented inulin slowly. The strain which failed to ferment after it was cultivated in the serum of the patient for one month fermented inulin after passage through a rabbit (see Table 1). The cultures isolated after death from the vegetations all fermented inulin slowly while those from the blood after death behaved the same as those isolated during life. It would seem as if the strains which did not ferment inulin had existed for some time in the blood of the patient, being probably descended from cocci washed into the circulation from the vegetations. Hence the inability to ferment inulin in a strain isolated from the blood in a single instance is not an argument against its being a pneumococcus.

Table 1 gives further observations with other strains of endocarditis cocci, with streptococci, Micrococcus rheumaticus, and pneumococci isolated from the blood of pneumonia. A glance at the table shows that the clotting of milk, the fermentation of saccharose, lactose, raffinose, salicin, and mannit, and growth or not on gelatin at 20° C. are of little value in differentiating streptococci from pneumococci. The length of time pneumococci are cultivated on artificial media determines largely whether they grow in gelatin or not. When first isolated they usually fail to grow. The fluctuation in the power to ferment inulin is very interesting. The endocarditic cocci usually ferment inulin slowly when first isolated; a short time later this power may be increased, only to disappear entirely after prolonged cultivation on blood-agar. The pneumococci from pneumonia at first ferment inulin strongly, but also lose it on long cultivation. If virulence is restored by animal inoculation the fermentative power returns. Streptococcus pyogenes and Streptococcus viridans, or "Streptococcus salivarius" of Horder, do not ferment inulin.

TABLE 1.

BIOCHEMICAL REACTIONS OF VARIOUS STRAINS OF PNEUMOCOCCI AND STREPTOCOCCI.

Strains of Pneumococci and Streptococci	Time Grown on Artificial Media	Milk Clotted	Acid in Saccharose	Acid in Lactose	Acid in Raffinose	Acid in Inulin	Acid in Salicin	Acid in Mannit	Growth in Gelatin at 20° C.	Character of Growth and Morphology in Broth	Green on Blood-Agar Plates
Pneumococci from endocarditis:											
Case 362. Strain isolated from first blood culture	2 days	±	+	+	+	+	o	—	o	Clumps in bottom; char. long chains	+
	3 months	+	+	+	+	o	+	o	+	Diffuse turbidity; diplococci	+
Case 362. Strain isolated in fifty-second blood culture	2 days	+	+	+	+	+	+	—	o	Long chains; clumps in bottom	+
Case 362. Strain from fifty-second blood culture after 5 rabbit passages	5 days	+	+	+	+	+	—	—	o	Diffuse turbidity; diplococci	++
Case 292	1 week	+	..	..	..	+	..	..	o	Long chains	+
Case 292	2 years	+	+	+	o	±	+	+	+	Diffuse turbidity	++
Case 293	5 days	±	..	..	..	+	..	..	o	Long chains	Slight
Case 293	2 years	±	+	o	o	o	+	+	+	Diffuse turbidity	+
Case M	2 weeks	+	—	—	—	+	—	—	+	Long chains	..
Case M	3 years	±	—	+	—	o	+	+	+	Diffuse turbidity	+
Case 409	1 week	+	++	+	o	+	+	—	+	Turbidity and clumps at bottom; short chains	+
Case 408	2 weeks	+	+	+	..	+	—	—	o	Diffuse turbidity; diplococci	+
Case 404	2 months	+	+	+	o	+	+	—	o	Clumps; no turbidity; long chains	+
Pneumococci from pneumonia:											
Case R	2 weeks	+	—	—	—	++	—	—	o	Diplococci	++
Case R	7 years	—	—	—	—	o	—	—	+	Diffuse turbidity; long chains	+
Case R2. Passages in rabbit	6 months	+	+	—	—	+	—	—	+	Diffuse turbidity; long chains	+
Case R3. Passages in rabbit	3 weeks	—	—	—	+	+	—	—	o	Long chains; capsules +	++
Case 245	Immed.	±	..	..	..	+	..	..	o	Diffuse turbidity; diplococci	++
Case 245	5 years	++	++	++	+	o	+	+	+	Diffuse turbidity; long chains	+
Case 245. After 4 passages in rabbit	2 weeks	+	+	+	+	+	—	+	o	Diffuse turbidity; diplococci; capsules	++
Case 210	Immed.	+	—	—	—	++	—	—	o	"	++
Case 210	5 years	+	+	+	+	o	+	+	+	Diffuse turbidity; long chains	+
Case 210. After 6 passages in rabbit	2 weeks	+	+	—	+	+	—	+	o	Diffuse turbidity; diplococci; capsules	++
Streptococcus viridans	6 months	+	+	+	+	o	o	o	+	Clumps; no turbidity; long chains	+
Streptococcus pyogenes	2 months	+	+	+	o	o	+	o	+	Diffuse turbidity; long chains	o Hemolysis
Micrococcus rheumaticus	$2\frac{1}{2}$ years	o	+	+	+	±	+	—	+	Diffuse turbidity; some chains	+

When first isolated the endocarditis pneumococci form long chains and clumps in broth, often growing in tenacious masses at the bottom of the tube and producing no turbidity. Sooner or later after cultivation they grow in diplococcus forms with occasional short chains, while still later they grow again in longer chains but now produce a diffuse turbidity. Marked involution forms are common as in the case of pneumoccoci from pneumonia which have been cultivated for a long time (see Fig. 1).

Fig. 1.—Smear from a 24-hour culture on blood-agar of pneumococcus cultivated on artificial media (chiefly on blood-agar) for seven years. Note long chains and involution forms. ×1,000.

The production of green by the endocarditis cocci on blood-agar when first isolated is always less than in those from pneumonia. In a number it was very slight when first isolated. Hemolysis about the colonies was never observed when fresh human blood was used in preparing the plates. After cultivation, as other characteristics of typical pneumoccoci return, the production of green is greater.

In this connection another point of importance must be mentioned. As stated, the effect of normal serum on these organisms is pronounced. Subcultures on blood-agar of cocci treated with serum repeatedly showed a greater production of green by those treated in normal serum than by those treated or "sensitized" in the serum of the patient. There was also marked difference in the growth of these organisms in normal serum and in patient's serum, and the injection of normal serum into the patient seemed to modify the organisms so that the production of green about the colonies on blood-agar became greater, chain formation less pronounced, and adherence to the surface of the agar less marked than before. In cases not injected with normal serum these characteristics seem to become more and more marked as the disease progresses. In a patient (Case 362, p. 429) who

received many intravenous injections of normal serum the gradual diminution of the tendency of the organisms to adhere to surfaces was associated with an unmistakable diminution in the agglutinating power of the patient's serum. The limit of agglutination before the injections were begun was 1:3,000. This gradually came down until after 18 injections of normal serum it was 1:150. The subcutaneous injections of smaller doses in Case 408 seemed to have a similar effect, altho not so great. Whether diminution in agglutination was due to a lessened agglutinability of the bacteria or disappearance of agglutinins from the blood was not decided.

If the organisms in question are modified pneumococci, then we would expect that as the cultural characteristics of typical pneumococci return, the original pathogenic properties should also return. I have shown elsewhere[1] that the adherence to the surface and growth in clumps are closely related to the ability of these bacteria to produce endocarditis in animals. The cocci from the animals that died with endocarditis showed the original "special" characteristics, while in animals which died soon after inoculation from pneumococcemia or from pneumonia, the cocci in general gave the characteristics of typical pneumococci. Exactly similar results, but on a larger scale, were obtained in the animals inoculated with the bacteria from the present series. The production of pneumonia in animals with cocci from cases of endocarditis and the isolation after death of typical pneumococci from the blood and from the consolidated lung would seem to furnish crucial evidence that the endocarditis cocci are pneumococci. By means of plate cultures I was able to assure myself that the bacteria injected consisted of so-called modified pneumococci. Further, if the "special" characteristics of the modified pneumococci are the result of long growth in the circulating blood, as seems probable, then the prolonged cultivation of typical pneumococci in endocarditis serum in the test tube might modify them in a similar manner. Three strains of typical pneumococci from the blood of patients with lobar pneumonia, grown in the filtered serum from Cases 408 and 409 for a period of three weeks, assumed long chains with involution forms much as the organisms from endocarditis; they also became more agglutinable and produced

[1] *Jour. Infect. Dis.*, 1909 6, p. 245.

in broth a sediment as well as a diffuse turbidity; fermentation of inulin was less marked, and the production of green on blood-agar diminished.

AGGLUTINATION TESTS.

As cases of chronic infectious endocarditis with joint involvement frequently resemble cases of rheumatic fever, agglutination tests were made in order to determine whether agglutination might be of any diagnostic value (Table 2). The results may be summarized as

TABLE 2.

Agglutination of Pneumococci, Streptococcus pyogenes, and Micrococcus rheumaticus by Sera from Cases of Endocarditis and Rheumatism.

Bacteria	Sera											
	Chr. Endoc. (408)		Chr. Endoc. (409)		Chr. Endoc. (413)		Ac. Pneu. Endoc. (412)		Ac. Rheum. (411)		Normal	
	1:20	1:1000	1:20	1:1000	1:20	1:1000	1:20	1:1000	1:20	1:1000	1:20	1:1000
Pneumococcus, chr. endoc. 293 (2 years)	+	o	++	o	++	o	—	o	o	o	o	o
Pneumococcus, chr. endoc. 209 (10 days)	+	o	+++	+	+++	o	++	o	o	o	o	o
Pneumococcus, chr. endoc. 413 (3 days)	++	—	++	—	++	+	—	+	+	—	+	—
Pneumococcus, chr. endoc. 362 (3 months)	+	o	+	o	+	o	—	o	o	o	o	o
Pneumococcus, chr. endoc. 292 (2 years)	++	o	+	o	+	o	—	o	o	o	o	o
Pneumococcus, ac. endoc. 412 (3 days)	+	o	+	o	++	o	+	o	o	o	o	o
Micrococcus rheumaticus ($2\frac{1}{2}$ years)	o	o	o	o	o	o	—	o	±	o	o	o
Pneumococcus pneumonia 410 (5 days)	o	o	+	o	++	o	o	o	o	o	o	o
Strept. pyog. (4 months)	o	o	o	o	—	—	o	o	+	o	o	o

The figures in parentheses in column headed by "Bacteria" give the time since the strains were isolated.

The bacteria used in these tests were grown in sugar-free dextrose broth for 24 hours and then heated at 60° C. for 30 m. The results were recorded after the mixtures had remained at 37° C. for 24 hours.

follows: The strains from my cases of chronic pneumococcus endocarditis were all agglutinated by the serum in each case. None of these sera agglutinated Micrococcus rheumaticus (Poynton and Payne) or Streptococcus. The serum of the case of acute pneumococcic endocarditis did not agglutinate Streptococcus or Micrococcus rheumaticus, but it agglutinated the strains of pneumococci from chronic endocarditis (as well as its own strain). The rheumatism serum agglutinated strain 413 (from chronic endocarditis) to the same extent as normal serum; it did not agglutinate the other pneumococcic strains, but agglutinated distinctly Micrococcus rheumaticus

and Streptococcus. It would seem then that in chronic infectious endocarditis agglutination tests may give results of some diagnostic value. My results strengthen the observations by Meakins,[1] Tunnicliff,[2] and others to the effect that rheumatism may be a streptococcus disease.

ANIMAL EXPERIMENTS.

I have shown that the production of endocarditis in animals by pneumococci without injuring the valves is closely dependent on special characteristics of the cocci and on the largeness of the dose. It was noted that while the first injection rarely caused lesions or death it rendered the animal more susceptible to subsequent inoculations, and that animal passage had a marked effect on the morphology and other characteristics of the organisms. Horder[3] produced endocarditis in rabbits by repeated injections of huge doses of non-virulent or "saprophytic streptococci" as he calls them. Others have had similar results. The possibility of producing endocarditis without trauma of the valves under proper conditions seems settled, but a closer study of the changes in morphology and pathogenicity of the bacteria inoculated seems desirable and the animal experiments given in Tables 3, 4, and 6 were undertaken for this purpose.

The development of only very few cases of endocarditis in this series as compared with the former is explainable because the number of bacteria inoculated was too small or the special characteristics were largely lost at the time of injection either by prolonged cultivation or as the result of the injection of normal serum into the patient.

Table 3 gives the results obtained from the injection of a single dose. They show a marked increase in "virulence" of the bacteria when cultivated in the patient's blood as compared with those grown on artificial media, quite independently of the place of inoculation. A single injection in all but one instance of even a few bacteria proved fatal (182, 187, 191, 194, 197, and 50). This virulence is also observed in animals which had recovered from a previous inoculation at the time of the second injection (Table 4). Continuous cultivation in fresh normal blood has a similar, but not so marked an effect (203, Table 3; 183, 52, Table 4). The primary injection of the bacteria

[1] *Can. Jour. Med. and Surg.*, 1909, 25, p. 71.

[2] *Jour. Infect. Dis.*, 1909, 6 p. 346.

[3] *Quart Jour. Med.*, 1909, 2' p. 289.

TABLE 3

Summary of Experiments on Rabbits with Single Injection of Pneumococci from Endocarditis.

No.	Inoculations: Date; Dose; Place	Time Since Organism Was First Isolated	Date and Cause of Death	Remarks and Postmortem Findings
182	1/8, Pn. 362.—4 blood-agar slants washed off in 3 c.c. of 24-hr. culture in patient's blood; intraperitoneally	4 days	1/9 Pneumococcemia	Blood crowded with typical lanceolate pneumococci. Subcultures show normal type pneumococci
187	1/28, Pn. 362^{15}*.—2 c.c. 362 blood after 48 hours' incubation intraperitoneally. No organisms seen in smears but cultures on blood-agar yield 10 colonies per c.c. Subcultures from all colonies yield modified pneumococci	2 days	1/12 Pneumococcemia	No anatomic lesions. Blood cultures on blood-agar yield moderate number of greenish colonies of typically lanceolate encapsulated diplococci, which are quite freely susceptible to phagocytosis by human leukocytes in human serum (see animal 188, Table 4)
191	2/17, Pn. 362^{41}.—24-hr. culture in 1 c.c. 362 blood (abundant growth) intraperitoneally. All subcultures from 10 colonies on blood-agar show modified pneumococci, but they no longer adhere to surface	2 days	2/25 Pneumococcemia	No gross lesions. Smears of bone marrow show many diplococci. Blood and peritoneal smears sterile. Blood cultures yield few green colonies of normal type of pneumococcus, susceptible to phagocytosis and not highly virulent (see animal 193, Table 4)
194	3/1, Pn. 362^{57}.—2 c.c. 362 blood in which pneumococcus was grown 48 hours intraperitoneally	3 days	3/27 Chloroformed	Marked emaciation; hemoglobin 55 per cent; no gross lesions; culture sterile
192*b*	2/27, Pn. 362^{51}.—2 c.c. 362 blood intraperitoneally (2 c.c. plated out at same time yield three colonies pneumococci all of modified type, but no longer adherent to surface)	½ day	4/11 Pneumonia	Marked loss in weight; pneumonia, acute tracheo-bronchial lymphadenitis. Pneumococci isolated from heart's blood and consolidated lung have characteristics of ordinary pneumococci. Moderately virulent altho quite susceptible to phagocytosis (see 219 below, and 185, Table 4)
189	2/17, Pn. 362^{41}.—5 c.c. 24-hr. blood broth culture intravenously, 5 c.c. intraperitoneally, and 5 c.c. subcutaneously	2 days	3/18 Pneumococcemia	No gross lesions. Cocci isolated of normal type
190	2/17, Pn. 362^{41}.—Same as 189 +5 c.c. normal human defibrinated blood intraperitoneally	2 days		Remained well
50	8/6, Pn. 408.—4 c.c. intravenously of 48-hr. ascites broth culture after cultivation in 408 blood for four weeks	4 weeks	8/26 Endocarditis	Endocarditis of tricuspid valve. Anemic infarct left lower lobe. Strain isolated resembles one injected
219	4/4, Pn. 192*b*.—2 c.c. 24 hr. broth culture and one blood-agar slant intraperitoneally	11 days	4/22 Pneumococcemia	No gross lesions. Organism isolated from heart's blood in large numbers and of normal type
203	3/24, Pn. 311^{8}.—After cultivation in normal human serum for three weeks; 5 c.c. 24-hr. milk culture and two ascites agar slants intraperitoneally	2 years	4/21 Infection	Marked emaciation. No peritonitis; no endocarditis but abscess of liver. Pus yields pure culture typical pneumococcus. Inoculation of agar-grown culture in another animal caused no lesions

* The figures to the right and above the number of the strain of pneumococcus indicate the blood culture in which the strain injected was isolated, i.e., 362^{15} indicates the strain isolated in the fifteenth blood culture in Case 362.

TABLE 3.—*Continued.*

No.	Inoculations: Date; Dose; Place	Time Since Organism Was First Isolated	Date and Cause of Death	Remarks on Postmortem Findings
55........	8/14, Pn. 408.—5 c.c. intravenously ascites broth culture of organism isolated from blood of 49, day after injection	5 weeks	9/7 Paralysis	Ill for several days. Then recovered. Died from ascending paralysis which came on four days before death. No anatomical lesions. No meningitis. Blood cultures negative except from spinal cord which yields a pneumococcus
195.......	3/6, Pn. 362^{57}.—10 c.c. 48-hr. broth culture into left ventricle	7 days	4/12 Pericarditis	Marked emaciation; huge adhesive and fibrinous pericarditis. Organism isolated from heart's blood and pericardium shows modified characteristic, but not adherent to surface of agar
196.......	3/3, Pn. 362^{57}.—10 c.c. broth culture into left ventricle	7 days	3/13 Endocarditis and pericar ditis	Fibrinous pericarditis; beginning endocarditis. Organism isolated in pure culture grows in clumps moderately adherent to surface
197.......	3/6, Pn. 362^{57}.—7 c.c. 24-hr. broth culture into right and left ventricle	7 days	4/1 Endocarditis and pericarditis	Endocarditis of aortic valve; mural endocarditis of left ventricle. Organism isolated resembles original culture closely. Adhesive and fibrinous pericarditis
201.......	3/22, Pn. 362^{8}.—Three blood-agar slants and three ascites agar slants intraperitoneally and into heart	63 days	4/20 Chloroformed	Had lost some in weight. Adhesive and fibrinous pericarditis. No other gross lesions. Heart's blood sterile
192.......	2/17, Pn. 362^{41}.—10 c.c. 24-hr. broth culture and one blood-agar slant intraperitoneally	2 days	3/15 Thrombosis of portal vein	No endocarditis but thrombosis of terminal radicles of portal vein. Smears from thrombus rich in diplococci. Cultures show modified pneumococci
205.......	3/24, Pn. 362^{8}.—10 c.c. ascites broth culture into heart and 10 c.c. intraperitoneally	65 days	4/19 Pericarditis	Fibrino- and fibrous pericarditis No other lesions. No endocarditis. No peritonitis. Cultures from heart's blood sterile. Smears from pericardium show modified pneumococcus but it does not adhere to surface of agar

isolated before the injections of normal serum in Patient 362 were begun, and cultivated on artificial media, never proved fatal either intravenously or intraperitoneally or both. After continuous cultivation in the patient's blood for four weeks (50) a single intravenous injection caused death from endocarditis. Injection of the bacteria, cultivated on artificial media for a considerable time, directly into the heart always produced pericarditis and usually also endocarditis. No increase in virulence could be detected in pneumococcus 362 after it showed definite reversion to the normal type, the result apparently

TABLE 4.

Repeated Injections of Rabbits with Strains of Pneumococcus from Endocarditis.

No.	Inoculations: Date; Dose; Place	Time Since Organism Was First Isolated	Date and Cause of Death	Remarks and Postmortem Findings
181	12/16, Pn. 362.—10 c.c. broth culture and one blood-agar slant intravenously; 10 c.c. intraperitoneally and subcutaneously	10 days	1/9 Pneumococcemia	Prompt recovery from first injection. Death in 36 hrs. after second injection. Pneumococci very numerous in blood, lanceolate, encapsulated, and subcultures give normal type pneumococci
	1/7.—Four blood-agar slants washed off in 3 c.c. of 24-hr. culture in patient's blood intraperitoneally	4 days		
183	1/11, Pn. 362^{4}.—6 c.c. 48-hr. culture in normal blood (growth abundant) intraperitoneally	7 days	3/4 Inanition; thrombosis of coronary veins	Ill for one or two days after first injection, then recovery. Very ill for three days after second injection, then gradually improved for a time, but later lost markedly in weight. Thrombosis right coronary vein. Organism isolated in pure culture from thrombus; heart's blood sterile; modified type of pneumococcus
	1/28, Pn. 362^{11}.—Three blood-agar slants intraperitoneally	2 days		
184	1/11, Pn. 362.—Five blood-agar slants washed off in broth intraperitoneally	27 days	2/22 Unknown	Seemed ill for day or two, then recovered both after first and second injection. Cultures from heart's blood and pericardial fluid sterile. No lesion.
	1/28, Pn. 362^{15}.—Three blood-agar slants +2 c.c. normal human blood intraperitoneally	2 days		
185	1/28, Pn. 362^{15}.—Three blood-agar slants intraperitoneally	2 days	5/5 Pneumonia	Ill for day then complete recovery. Recovered more promptly than 184 after second injection, nor was illness as great (see 192, Table 10)
	2/17, Pn. 362^{41}.—10 c.c. ascites broth + one blood-agar slant + 2 c.c. normal human blood intraperitoneally	37 days		
	192*b*.—3 c.c. 24-hr. broth culture and one blood-agar slant intravenously	10 days		Apparent recovery for six days after third injection, then began to breathe rapidly, etc. Pneumonia, acute peribronchial lymphadenitis. Cultures from blood sterile; from lung encapsulated diplococci of normal pneumococcus type
188	2/14, Pn. 362^{15}.—Two agar slants of organism isolated from animal 187, Table 10, intraperitoneally	2 days	4/22	Prompt recovery after severe illness for a day. Severe illness following second injection but finally recovered. Seemed perfectly well when injected third time. Severe illness due to large dose directly into heart. No hypersensitiveness. Death from pneumococcemia in two days after third injection. Healing serofibrinous pericarditis. Blood cultures yield diplococci with short chains.
	3/24, Pn. 362^{1}.—10 c.c. 48-hr. ascites broth culture into heart	65 days		
	4/21.—10 c.c. intraperitoneally. 3 c.c. broth culture Pn. from rabbit 192, and surface growth of blood-agar slant, intravenously	10 days		
193	3/1, Pn. 362^{41}.—After passage through animal 191, three blood-agar slants intraperitoneally	4 days	3/27 Chloroformed	Very ill after first injection but prompt recovery after second injection. Blood cultures after death sterile
	3/6, Pn. 362^{57}.—3 c.c. of 48-hr. blood-broth culture intraperitoneally	7 days		
49	8/6, Pn. 408.—5 c.c. ascites broth culture after cultivation in 408 blood for four weeks, intravenously	4 weeks	9/7 Chloroformed	Day after injection 250 pneumococci per c.c. of blood. Very ill for short time but recovered completely. No lesions. Blood culture sterile
	8/14, Pn. 408.—The same as above after cultivation on blood-agar for five days	5 weeks		

TABLE 4.—*Continued.*

No.	Inoculations: Date; Dose; Place	Time Since Organism Was First Isolated	Date and Cause of Death	Remarks and Postmortem Findings
48	8/3, Pn. 408.—6 c.c. ascites broth culture intravenously 8/14, Pn. 408.—5 c.c. ascites broth after cultivation in 408 blood for four weeks and on blood-agar five days, intravenously	4 weeks 5 weeks	9/7 Chloroformed	Not as ill as 44, Table 10, which had received Pn. 409 three weeks previously; 250 pneumococci per c.c. of blood. Control dead fourth day. Complete recovery. Very ill for two days then improved five days after injection. Arthritis of left front ankle joint. Recovered. Autopsy: No lesions; blood sterile.
52	8/6, Pn. 408.—4 c.c. ascites broth culture after growth in normal blood for 4 weeks, intravenously 8/14, Pn. 408.—5 c.c. ascites broth culture after cultivation in normal blood for 4 weeks, and on blood-agar 5 days, intravenously	4 weeks 5 weeks	8/16 Pneumonia	Recovered promptly after first injection. Very ill after second. Death two days after injection from beginning pneumonia. Cultures from heart's blood and lung yield typical pneumococci

of numerous intravenous injections of normal human serum in the patient.

Normal serum being found to raise the phagocytic and destructive power of the patient's blood to the normal point when low, it is interesting to note that the subcutaneous injection of normal serum into the infected rabbit served to protect the animal from otherwise fatal doses. This was found to be true for the first as well as subsequent inoculations (184, 185, Table 4; 190, Table 3).

The aforementioned increased susceptibility of animals to subsequent injections of these organisms is confirmed by the results obtained in the present study (Table 4). The hypersensitiveness is roughly proportionate to the dose, but quite independent of the place of inoculation, i.e., intraperitoneal injections sensitize to subsequent intravenous injections and vice versa. In order to produce death promptly after a second injection relatively large doses are necessary. The seemingly increased susceptibility to infection might in reality be an expression of an active immunity and death the result of an overwhelming intoxication from rapid destruction of the organisms injected. In regard to this point there is this to say, that the "sensitized" animals present none of the usual symptoms of anaphylaxis following the second injections—the reaction is different from the

anaphylactic phenomenon following the injection of foreign proteids. Moreover, a study of peritoneal smears during life, instead of showing a more rapid phagocytosis and destruction of cocci after the second

TABLE 5.

Sensitization of Rabbits with Pneumococci from Endocarditis.

No.	Inoculations: Date; Dose; Place	Time Since Organism Was First Isolated	Date and Cause of Death	Remarks and Postmortem Findings
44	7/9, Pn. 409.—Washed and suspended in NaCl after growth in 3 c.c. 409 serum for 48 hours; 2 c.c. intravenously; 1 c.c. intraperitoneally 8/3, Pn. 408.—6 c.c. ascites broth culture intravenously (see 48, Table 4, the control)	4 days 4 weeks	8/5 Pneumonia	Ill for only day or two after injection of Pn. 409; complete recovery. Day after second injection, animal showed dyspnea and 5,600 pneumococci per c.c. of blood. Lobar pneumonia, stage of red hepatization; beginning pericarditis; no endocarditis; no arthritis. Typical pneumococci from heart's blood and consolidated lung (see plate)
43	7/9, Pn. 409.—2 c.c. intravenously and 1 c.c. intraperitoneally of 409 serum in which Pn. 409 was grown for 48 hrs. (see 44) 8/6, Pn. 408.—5 c.c. ascites broth culture after cultivation in 408 blood for four weeks, intravenously (see 49, Table 4) 8/14, Pn. 408.—8 c.c. ascites broth culture after cultivation in 408 blood for four weeks and then on blood-agar five days, intravenously	4 days 4 weeks 5 weeks	9/7 Chloroformed	After first injection seemed ill for two days, then recovered. Blood cultures two days later sterile. Seemed very ill day after second injection. Breathes rapidly; 13,000 whites; 180 colonies pn. per c.c. of blood. Rapid breathing kept up for five days longer, then recovered. Recovery after third injection prompt. Blood cultures day after sterile. Autopsy: No lesions; blood cultures sterile
4	7/16.—3 c.c. broth culture intravenously of pneumococcus isolated from pneumonia in man 8/8, Pn. 409.—6 c.c. ascites broth culture intravenously	1 week 2 months	9/7 Chloroformed	Prompt recovery after first injection. Ill for a time after second injection. No evidence of hypersusceptibility. Autopsy: Healed adhesive pericarditis; otherwise normal; blood sterile
45	7/7, Pn. 412.—(From acute pneumococcus endocarditis) 6 c.c. ascites broth culture + growth on 3 blood-agar slants, intraperitoneally 8/9, Pn. 409.—6 c.c. ascites broth culture intravenously	7 days 3 months	9/7 Chloroformed	Ill for short time, then complete recovery. First injection did not render animal susceptible to second injection. Recovery complete. No loss in weight. Autopsy: No lesions; blood sterile
47	7/7, Pn. 410.—8 c.c. ascites broth culture from pneumonia intravenously 8/9.—6 c.c. intraperitoneally of ascites broth of Pn. 412 from case of acute pneumococcus endocarditis	3 weeks 2 months	9/7 Chloroformed	Ill for a time, then complete recovery No evidence of increased susceptibility. Prompt recovery. Autopsy: No lesion; culture sterile

or third injection, show an actual diminution in phagocytosis and a slower destruction than after the first injection. Again, cultures show actual multiplication of organisms in both the animals dying

promptly from pneumococcemia without localization as well as in those which died later from endocarditis and pneumonia after a second injection. These facts would seem to prove that death must be due to increased susceptibility to infection.

It is a well-known fact that injection of pneumococci from pneumonia and other sources may produce an increased resistance to subsequent injections. Prolonged cultivation on artificial media, a short residence in normal serum, and animal passage, as already pointed out, have a pronounced effect on the morphology and cultural characteristics of these organisms. They become more like pneumococci from pneumonia. See Figs. 2, 3, 4, 5. Table 4 shows that prolonged cultivation on artificial media and cultivation in normal serum reduce the sensitizing power of the organisms to a definite degree, while animal passage takes away nearly all this property (184, 188, 193, 49, 48). In other words, reversion in morphology to the normal type occurs with the return of original pathogenic properties. In this connection there is still another point of interest. If these strains of pneumococci coming from different cases of endocarditis, and showing degrees of modification, belong to the same group, then we should expect that the injection of one strain would render the animal hypersensitive to the other strains also. In Table 5 it is shown that this is true. Moreover, the increased susceptibility is found to extend to moderately virulent pneumococci from pneumonia and from acute pneumococcus endocarditis. This fact is in accord with the idea that the strains studied are modified pneumococci.

Against the idea that in the animals dead from pneumococcemia or pneumonia in which the cocci isolated after death resembled typical pneumococci I was dealing with a mixt culture, it must be stated that this point was carefully controlled by plating out part of the material at the time of inoculation and studying the organisms from the individual colonies (187, 191, 192*b*, Table 3). In each instance only modified pneumococci were found. Similar observations were made of the colonies obtained on the blood-agar plates from the blood cultures obtained from the patients. In no instance were typical pneumococci isolated. Furthermore, the postmortem findings in Cases 362 and 408 (pp. 429, 431) showed no lesions which would lead one to suspect pneumococci of the normal type in the circulating blood

FIG. 2.—Typical encapsulated diplococci in smears of blood of rabbit dead from injection with modified pneumococcus of endocarditis. ×1,000.

FIG. 3.—Pneumococcus 409 grown in serum 409 for 10 days. Note the small size of the cocci and the long chains. The cocci in the chains appear as diplococci. ×1,000.

FIG. 4.—The same strain as Fig. 3 cultivated on blood-agar for 48 hours and in normal blood for three days, transfers being made daily. Note absence of chains and the larger size of the cocci which are often lanceolate in shape. ×1,000.

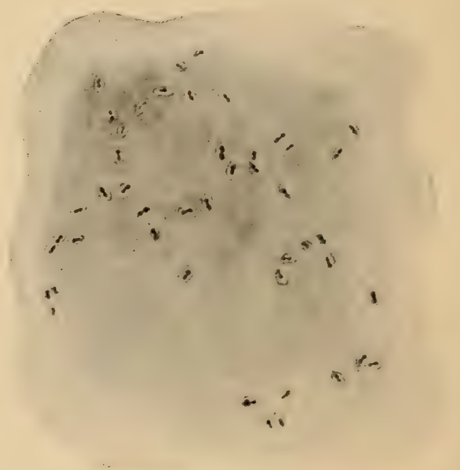

FIG. 5.—Buerger's capsular stain of pneumococci in rabbit blood after death from inoculation with pnuemococcus in Fig. 1 treated with extract of virulent pneumococci. ×1,000.

at the time the animal experiments were made. Spontaneous infection from outside sources is inconsistent with the results obtained in the animals which died of pneumococcus pneumonia. The animals were caged separately and no epidemic occurred among the other animals at the time of the experiments.

A study of the peritoneal smears in the animals injected intraperitoneally for the first time with cocci grown artificially showed marked phagocytosis and an exceedingly rapid destruction of the

FIG. 6.—Experimental endocarditis in rabbit. Normal size. Three weeks before death the rabbit was given an intravenous and intraperitoneal injection of pneumococcus 408 after cultivation in the patient's serum for four weeks. The valves were not previously injured.

bacteria, the animals recovering promptly. When injected the second or third time with the same cocci or the first time with those grown in the patient's blood, both phagocytosis and destruction, while much delayed, were still pronounced, and the animal either succumbs in a short time from pneumococcemia or pneumonia, or later from endocarditis or pericarditis.

After death, smears from the blood, the pericardium, and the vegetations showed remarkably little phagocytosis; the organisms isolated from the blood or from the lesions, while quite susceptible to phagocytosis by human leukocytes in human serum, were not so freely taken up as previously and showed a corresponding increase in virulence, which was promptly lost on artificial cultivation. The strains isolated from those which died from endocarditis or peri-

carditis were more freely taken up than those dead from pneumococcemia. No difference could be established in this respect between those isolated from the animals dead from a primary or subsequent injection.

The morphology of the cocci isolated after death on the one hand, and the character of lesions produced on the other, show a striking

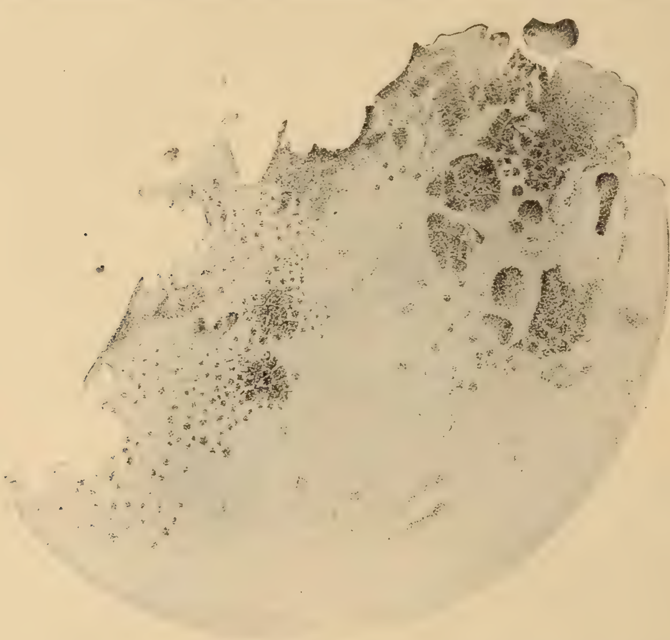

Fig. 7.—Gram strain of a section of vegetation from experimental penumococcus endocarditis in rabbit. The dark areas are collections of pneumococci imbedded in a fibrinous network. Their number and size increase as the free surface is approached. ×100.

parallelism. Thus in 13 out of 20 instances in which this point was studied the pneumococci isolated resembled closely typical pneumococci (see Fig. 2). Seven of these died of pneumococcemia without localization and one of pneumonia, one of abscess of the liver, and one of an ascending paralysis (55, Table 3). This animal was given a single injection of organism 408 after one animal passage. Of the remaining seven in which death occurred later, the organisms resembled more or less closely the ones injected; five showed endocarditis or pericarditis, or both; two thrombosis, one of the coronary artery and the other of the portal vein.

The specific affinity of these organisms for vascular endothelium, endocardium and pericardium especially, is striking. Peritonitis never developed, notwithstanding the fact that the injections were made intraperitoneally in most of the animals. Arthritis developed in only one animal (48, Table 4), from which it recovered. Subcutaneous abscesses never developed at the site of inoculation. It is interesting to note that pneumonia developed late (10 to 40 days) in the two rabbits receiving only one dose and promptly (2 days) in the two in which it developed after the second injection. The consolidation was not always distributed in lobes but the line of demarkation was sharp and the microscopic sections show the cellular infiltration characteristic of fibrinous pneumonia.

SUMMARY.

The reasons, then, for looking on the cocci isolated from the cases studied as modified pneumococci may be briefly summarized as follows: (1) The chains are made up of distinct elongated diplococci, often lanceolate in shape, frequently showing involution forms similar to those of ordinary pneumococci cultivated on blood-agar for a long time. (2) They always produce a variable amount of green on blood-agar plates, but usually much less than the typical pneumococci, and never hemolysis. (3) All the strains ferment inulin. (4) Colonies when first isolated adhere more or less closely to the surface of blood-agar slants and grow in clumps at the bottom of test tubes of broth or other liquid media and in the fibrin clot of the blood cultures, leaving a clear fluid. Sooner or later after cultivation on artificial media these special characteristics are lost; they no longer form chains, but are typical diplococci instead; no longer adhere to the surface of agar, and grow with a diffuse turbidity in broth. The more marked these characteristics are in the beginning, the longer it takes for them to disappear. (5) Cultivation in normal serum or blood and animal inoculation may call forth promptly the usual pneumococcal characteristics, i.e., typical lanceolate diplococci, often encapsulated, which produce more green on blood-agar and otherwise grow exactly as pneumococci from pneumonia, often result, as observed also by Buerger and Ryttenberg.[1] Recultivation in the patient's

[1] *Jour. Infect. Dis.*, 1907, 4, p. 609.

serum brings back the "special" characteristics, which is true also of strains which have lost them by cultivation on artificial media. (6) The special characteristics show great variations in the different strains studied. The more chronic the course of the disease, the more marked they are, and vice versa. In Cases 362 and 408 they were pronounced, while in Case 409, which ran a more acute course, they were less marked. (7) Reversion to the normal type, morphologically and culturally, is associated with return of the pathogenic properties more or less characteristic of typical pneumococci. (8) The pneumococcal organisms are agglutinated by the sera from the endocarditis cases in high dilution; and the sera also agglutinate typical pneumococci, but not streptococci. (9) Cultivation of pneumococci from pneumonia in fresh serum from cases of chronic endocarditis produces changes similar to those observed in the cocci isolated from the blood of cases of chronic infectious endocarditis.

IMMUNOLOGICAL STUDIES IN CHRONIC PNEUMOCOCCUS ENDOCARDITIS.*†

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INTRODUCTION.

In a previous paper on endocarditis[1] I have tried to show that the continuation of the infection and death are largely due to a process of bacterial immunization against the antibodies of the host rather than to the virulence in the usual sense of the infecting bacteria. It was hoped at the outset of the present study that frequent determinations over a long period, in suitable cases, of the opsonic index, of the antibacterial power of the patient's blood, and of the number of bacteria in the blood in relation to the clinical course and to the injections of dead bacteria and human serum, would throw some additional light on the problems in question.

The Cases.

Case 362.—M. J., age 52, lumberman, was seen by Dr. Billings, with his physicians, Dr. McKechnie and Dr. J. L. Miller of Chicago, in November, 1908, to whom I am indebted for the opportunity of studying the case.

The patient[2] had been a strong and healthy man all his life, whose business cares were great. He was addicted to the overuse of alcoholics and tobacco. Off and on for several years he had had various muscular ailments and disturbances of digestive organs. During the summer of 1908 he suffered from pyorrhea alveolaris and abscesses of the gums especially about the right upper second bicuspid tooth and also the left upper first molar, for which he received treatment from his dentist. During the late summer of 1908 the gums and teeth were in bad condition, many teeth hung loose. In July, 1908, he noticed lessened strength and endurance, at times he was chilly, and the temperature on a few occasions was found as high as 100. There were transient attacks of pain and some swelling in the various joints, especially of the feet. In the early part of November, 1908, a slight swelling and tenderness in the right great toe led to the diagnosis of gout. From this time onward there was a gradual loss of strength with headache and a temperature ranging from 98 to 99 in the morning to 100 to 101½ in the evening. In the latter part of this month the blood showed 11,000 leukocytes. An agglutination test with typhoid bacilli was said to have given positive results, and the

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[1] *Jour. Infect. Dis.*, 1909, 6, p. 245.

[2] The histories of this and the following case are largely taken from the article by Dr. Billings, "On Chronic Infectious Endocarditis," *Arch. Int. Med.*, 1909, 4, p. 409.

patient was placed on typhoid orders and treated as a typhoid patient for two or three weeks. He then suffered a sudden left hemiplegia and a sudden rise of temperature to 106. The temperature soon dropped to the former range, the hemiplegia improved,

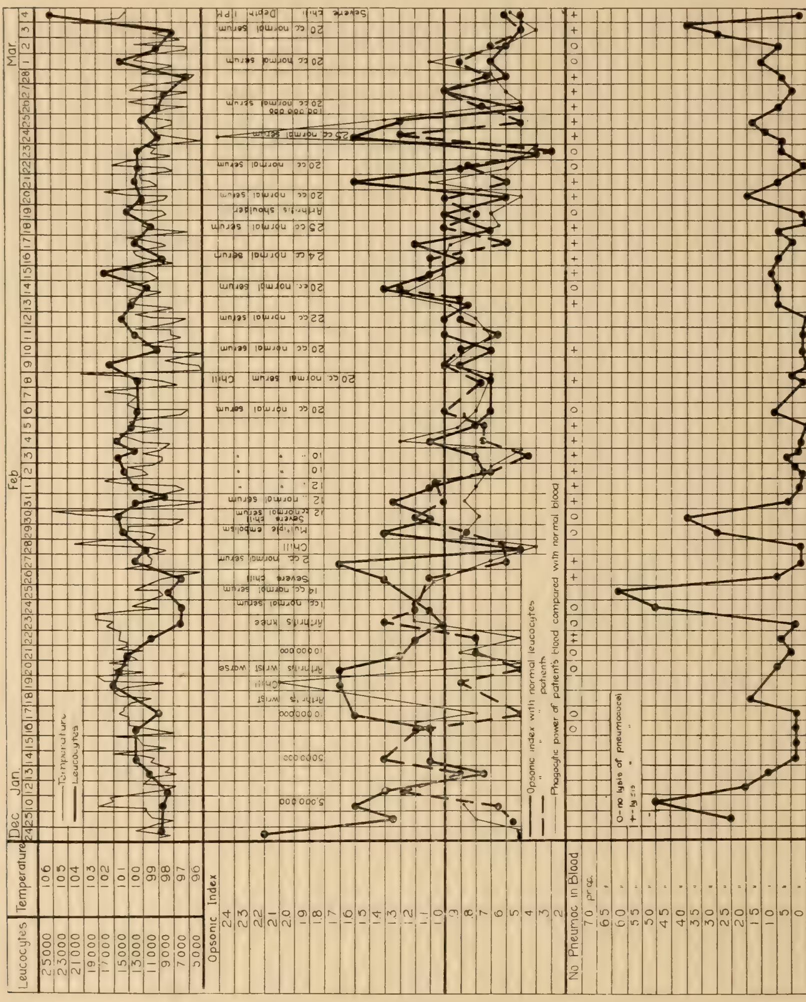

Chart 1 (Case 362).

and the patient returned to much the same condition as before. The examination of the heart up to this time had given entirely negative results, but from now on a systolic murmur was heard occasionally which later became permanent. Early in December the patient presented a septic type of temperature (see Chart 1), a slight left hemiparesis; he answered questions slowly and took little interest in his surroundings;

there was dilatation of the left heart and now a mitral systolic murmur was detected with transmission to the left as well as accentuation of the second pulmonic tone; lungs normal; liver palpable and somewhat tender; spleen not palpable; a few petechial spots were found in the skin in various parts of the trunk and extremities. The first two blood cultures were sterile, but the third and fourth and the remaining cultures, 53 in all, yielded a modified pneumococcus. On December 13 the Hb. was 85 per cent, the reds 4,000,000, the whites 17,700 with a preponderance of polymorphonuclears. On February 22, 1909, the Hb. was 70 per cent, reds 3,200,000 and the leukocytes 12,000.

Careful chemical analyses of the urine were made by Dr. S. A. Matthews, with, in brief, the following results. A small amount or traces of albumin were constantly present during the last month of the patient's life. The urea at times was increased but averaged 1½ per cent. The urine showed no increase in ammonia or creatinin; on the whole there was a slight diminution in creatinin. From the middle of January till death there appeared periodically variable amounts of a reducing substance which at first failed entirely to respond to carefully controlled fermentation tests in spite of the fact that the gravimetric method showed an average of 1 per cent glucose. Later the amount of glucose ranged between 2.8 to 3.8 per cent and the fermentation test now gave positive results. Tests for acetone, diacetic acid, and B-oxybutyric acid were uniformly negative. No definite relation could be established between the periodic excretion of sugar and the injection of human serum with the resulting reaction (see Chart 1).

Extract of postmortem record (Dr. D. J. Davis): A limited examination only was permitted after the body had been embalmed.

Anatomic diagnosis.—Subacute ulcerative mitral endocarditis; multiple infarcts of the spleen and kidneys; echymosis in the skin; hyperplasia of the spleen; healed and calcified tuberculosis of both pleurae; sclerosis of the aorta; obesity; ossification of the costal cartilages. The spleen soft, enlarged, with several anemic infarcts. The capsule of the kidney strips readily, leaving a smooth surface with two well defined infarcts in each, surrounded by a narrow red zone; the cortex swollen and everywhere studded with small fibrous and calcified nodules from 2 to 3 mm. in diameter. A few occur beneath the pleura in the lung tissue. The heart is large and at the base is a large amount of subpericardial fat. The muscle is firm (probably from the formalin). The pulmonary, tricuspid, and aortic valves are normal and the ascending and transverse aorta show small areas of sclerosis, non-calcified and diffuse. No sclerosis exists about the coronary openings. On both flaps of the mitral valve are large irregular gray masses about 2 cm. across and projecting outward into the lumen. They are firmly adherent to the valve and are covered by dark red blood clots.

Microscopic examination.—There are microscopic miliary abscesses in the liver, pancreas, heart muscle, and spleen. The number of leukocytes, especially polymorphonuclears, is markedly increased in the capillaries of all the organs. There are healed tubercles in the pleura, associated with considerable thickening and slight emphysema and bronchopneumonia. There is considerable fatty change of the liver cells and some round cell infiltration in the connective tissue. There is a marked increase in the cellular elements of the spleen associated with the large number of leukocytes.

Case 408.—A. K., man, married, age 30. I am indebted to Dr. Frank S. Johnson and Dr. Frank Billings for the facts in this case.

During childhood he had acute rheumatic fever with consequential mitral insuffi-

ciency and aortic insufficiency. Compensation occurred and the patient did not suffer until his twentieth year. During his first year at college he attempted rowing, track running, etc., but the result was broken compensation. After a prolonged rest compensatory hypertrophy was restored and he remained in fair condition until November, 1908, when he was operated on for acute appendicitis. He recovered promptly, but several months later there were lessened strength and endurance, a good deal of nervousness and irritability, slight shortness of breath on exertion, with some palpitation of the heart and some fever. Chills followed by fever occurred at irregular intervals. The spleen became palpable, he grew pale, had great dyspnea and palpitation of the heart. A septic type of temperature and a secondary anemia with a low white blood count developed. In May, 1909, the patient was pale, weak; a few petechial spots were found in the skin of the back; the heart was dilated, especially to the left, and the heart action was rapid; a soft blowing diastolic murmur was detected in the aortic area, transmitted down the sternum, and also a loud systolic murmur in the mitral area, transmitted to the left, with an accentuation of the second pulmonic tone. The lungs were clear, the spleen palpable, the liver not enlarged. The urine was practically normal. The blood on May 29 showed 78 per cent Hb., 4,228,000 reds and 8,000 whites. Except for a period early in July when there was a slight increase in Hb. coincident with a decided improvement in the patient's general condition, after the first injection of normal serum, the secondary anemia grew steadily more marked. August 3, 1909, four days before death, the Hb. was 40 per cent and the reds 3,128,000. Two blood cultures during the latter part of May were sterile, while all the subsequent cultures, 37 in all, gave growths of modified pneumococci (see curve in Chart 2). On June 16, 1909, bloody urine was passed; the blood soon disappeared and the urine remained normal until August 5, when blood again appeared, and in addition a large number of hyalin, granular, epithelial, and blood casts. In this case (as in others) the tendency to periodicity of the infection was marked. The occurrence of petechial spots, infarcts of the spleen, kidney, and of several attacks of cerebral embolism, was always associated with prostration, high fever, malaise. A wave of improvement lasting for a variable period occurred after each of these events, the strength, however, being less than before the attacks. This was likewise the case after the injections of dead pneumococci or serum or both combined. For 24 hours after such injection the patient showed signs of intoxication, especially when the bacterial count dropped markedly, but subsequently the patient felt improved. The large doses of pneumococci (200,000,000), given against my judgment, unfortunately exhausted the patient greatly and were not followed by the improvement seen after the smaller doses of cocci alone or serum and cocci together. These large injections seemed to overwhelm the recuperative powers (see Chart 2). The smaller dose, 50,000,000, together with 10 c.c. of normal serum given four days later, had the usual but temporary good effect (July 21 and 22).

A cerebral embolism causing a complete left hemiplegia occurred July 31, four hours after the subcutaneous injection of 10 c.c. of normal serum and 50 million pneumococci. During the night of August 3, four days before death, the patient's respirations grew more rapid, the pulse more bounding, and the temperature higher. This no doubt marked the development of the lobar pneumonia, from which the patient died, August 7, 1909.

Extract from postmortem report.—Anatomical diagnosis: Chronic aortic and mitral and acute ulcerative and vegetative aortic and mural endocarditis; recent infarcts of the spleen and kidneys; acute splenic tumor; lobar pneumonia; acute

fibrinous pleuritis; acute serofibrinous pericarditis; passive congestion and cloudy swelling of the liver; acute nephritis, hypertrophy of the left ventricle (marked); anemia; petechiae; laparotomy scar.

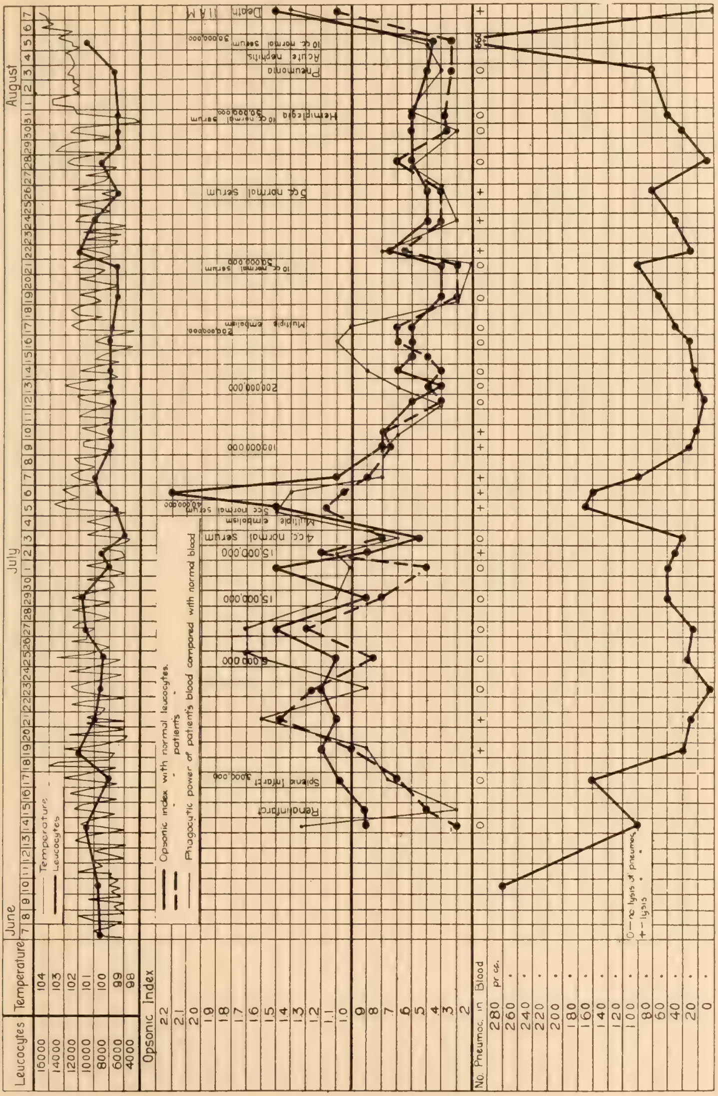

CHART 2 (CASE 408).

The body is that of a well-developed but emaciated young man. The skin shows hemorrhagic areas, varying in size from 2 to 18 mm. in diameter, the largest on the anterior and right side of the abdomen.

The pericardial cavity contains approximately 100 c.c. of a serous fluid in which are small fibrinous masses. The pericardium is smooth and shining. The wall of the left ventricle is much hypertrophied. The endocardium of the right side of the heart is normal. There is a much-thickened calcareous area at the base of the posterior aortic cusp. Several smaller areas are present at the base of the other two cusps. The aortic cusps show no loss of substance and are of normal shape, but are greatly thickened, especially along the free margin, in which there are several calcareous nodes. Growing from the free margins of these cusps are numerous pedunculated vegetations measuring 2–13 mm. in length, all but two springing from the ventricular side. Running down from the posterior cusp there is a large area of ulceration extending over the mural endocardium which covers most of the posterior surface of the posterior leaflet of the mitral valve. This area is 4½ cm. long and 2 cm. in its greatest width. Its base and margins are beset with numerous vegetations, all tending to become pedunculated. Some measure 2 cm. in length. Another smaller but similar area of ulceration is found near the base of the anterior leaflet. It extends downward for a distance of 2 cm. Numerous small areas of ulceration covered with vegetations are found upon the endocardium in the left ventricle directly opposite the large area of ulceration described above. The mitral orifice admits easily three finger-tips, the leaflets are thickened.

The pleural cavities are empty and free from adhesions except for a few over the base of the left lung, which is bound loosely to the diaphragm. The visceral and parietal pleurae show no changes except over the right middle and lower lobe, where it is opaque and rough with a small deposit of fibrin. The whole of the left lung and the upper lobe of the right crepitate throughout. Several depressed feebly crepitant areas are found in the left lower lobe posteriorly. The right lower lobe and nearly all of the middle lobe are dark grayish red in color, firm, and solid. The cut surface is mottled grayish red. There exudes a bloody fluid containing small fibrin plugs. Portions cut from both lobes sink in water.

The spleen contains not less than ten typical anemic infarcts, all hard but friable and surrounded by an area of hyperemia. There are two firm anemic infarcts in the right kidney. The liver was not examined.

Bacteriologic examination.—Smears made from both the right middle and lower lobes show a large number of typical lanceolate encapsulated, gram-positive diplococci, all outside the numerous leukocytes present. Blood agar plates from this material gave a rich pure culture of small colonies surrounded by an abundant greenish zone, markedly different from the zone of green produced by the organism isolated repeatedly from the blood during life. A pure culture of a similar coccus developed in broth. The diplococcus is lanceolate, encapsulated and does not form chains; it is grampositive. It is not susceptible to phagocytosis in normal and in the patient's blood, and when moderate doses were inoculated into a rabbit and guinea-pig it produced a fatal pneumococcemia in 24 hours. No bacteria in peritoneal or pericardial fluid.

The vegetations, after thorough washing in NaCl solution, and on being crushed with a sterile glass rod in this solution, give a moderate number of small gram-positive diplococci in short chains; the leukocytes are fairly well preserved, especially those which do not contain cocci; those which contain diplococci often show a marked disintegration, especially if the ingested organisms show disintegration. A few larger typical lanceolate gram-positive diplococci are also seen, none of which appear in

chains or within leukocytes. Cultures on blood agar plates show a moderate number of typical green-producing colonies, consisting of gram-positive large diplococci, and a much larger number of smaller colonies producing only a trace of green and consisting of small cocci like those isolated so frequently during life. They are freely susceptible to phagocytosis in normal and in the patient's blood.

The heart's blood gave sterile smears and cultures; one typical pneumococcus colony developed on blood agar in approximately 5 c.c. of blood.

Smears from the splenic pulp and infarction show no organisms. Cultures from the pulp result in the isolation of a pure culture of typical pneumococcus, 50 colonies developing upon blood agar plates per drop of pulp.

Case 409.—Salesman, age 26, single, admitted to the service of Dr. Billings at the Presbyterian Hospital, June 4, 1909. Two attacks of acute articular rheumatism 14 and 12 years ago; the heart was involved in both and for a time he was short of breath. Gradually compensation occurred, but he was "short winded" and could not endure the severer athletic sports while at college. Early in April increasing weakness, shortness of breath, and chills followed by fever and sweats came on. The diagnosis of rheumatism was made on account of an attack of pain in the left knee joint and of painful and tender spots with redness and some swelling in the tips of the fingers. Salicylates and a course of injections of mercury previous to entering the hospital failed to relieve the symptoms.

On admission there were marked pallor, dyspnea on slightest exertion, throbbing of vessels of the neck; tonsils of normal size, throat normal; moderate cyanosis, dry hacking cough; regular but quick almost "water-hammer" pulse, heaving precordial impulse. Left border of heart 13 cm. to left of median line; apex diffuse in sixth interspace. Right border 2 cm. to right of sternum. Churning systolic and diastolic murmurs. No edema of hands or feet. Lungs and pleurae free. Occasional petechial hemorrhages in the skin and painful nodes in tips of fingers.

The patient rapidly grew worse, became more and more short of breath and cyanotic; the precordial dulness increased. The day before death the left border and apex impulse were found in the mid-axillary line; edema of the feet, hands, and lungs gradually increased but the pleural cavities remained empty. No demonstrable ascites at any time. The patient died from exhaustion 18 days after admission.

The urine was scant, highly colored, remained free from blood and casts throughout; a trace of albumin was found on two occasions, while two other specimens showed none. The blood showed 4,240,000 reds, 9,600 whites, Hb. 53 per cent, and color index 6. The blood cultures in this case, six in all, each yielded from 30 to 532 colonies of pneumococci per c.c. of blood. The coccus was freely and equally phagocytable by normal and by patient's blood. The destructive power of the patient's blood was far below normal on two occasions within four days of death. The number of pneumococci in the blood at that time was large.

Extract from postmortem report.—Anatomical diagnosis: chronic and acute endocarditis of the mitral and aortic valves; infarcts of spleen and lung; edema of lungs; hypostatic pneumonia of right lower lobe; petechial hemorrhages; passive congestion of liver and kidneys; hypertrophy and dilatation of heart (cor bovinum); cloudy swelling of liver, kidneys, and myocardium; healed tuberculosis of lungs; anemia; acute peribronchial and mesenteric lymphadenitis.

The heart weighs 850 grams. The apex is made up entirely of the left ventricle. There are vegetative growths on both the mitral and aortic valves, grayish red in color,

the cusps being largely destroyed; the remnants, especially of the aortic valve, are fibrous and calcified. The growths spring from the thickened calcareous and fibrous areas of the valves.

Bacteriology.—Smears and cultures from the heart's blood, the vegetations of aortic and mitral valves, the peritoneal fluid, and splenic infarct yield a pure culture of pneumococcus similar to the ones obtained from the blood during life.

This organism is freely susceptible to phagocytosis when grown in broth by normal and patient's blood. It is non-virulent to animals (rabbits and guinea-pigs). When first isolated it forms long chains; grows in the fibrin clot of the blood cultures in large colonies; adheres slightly to the surface of agar and very closely in the colonies so that it is difficult to make even suspensions in salt solution, properties which are soon lost when cultivated on artificial media, when it soon grows like the typical pneumococcus. The organism invariably produces green on blood agar, never hemolysis, ferments inulin slowly, grows slightly in gelatin at 20° C.; acidifies and coagulates milk.

Plate cultures from a small area of consolidation in the lung yield a large number of the pneumococci above described, a few colonies of Strept. pyogenes and Staph. albus. Smears from the vegetations show that they are made up largely of diplococci and short chains and leukocytes held together by a fibrinous network. The appearance, except for the presence of leukocytes, is not unlike the smears made from the large masses which grow at the bottom of broth inoculated with these organisms. Most leukocytes contain no bacteria. Occasionally some are found which contain a large number. The leukocytes thus engaged in phagocytosis show marked disintegration while those which show none are well preserved. No lymphocytes and only few endothelial cells can be found. No eosinophiles.

Technic.

Blood cultures and bacterial counts.—At first the blood cultures in each case were made by drawing from 10 to 20 c.c. of blood from the vein at the bend of the elbow and planting it in liquid media and agar. After a sufficient number of cultures were made to establish the diagnosis and identity of the bacteria, later cultures to determine the number of bacteria were made from the lobe of the ear. The latter method is reliable for estimating the number of bacteria circulating in the peripheral blood in the cases studied, because in 15 simultaneous cultures from the vein at the elbow the same average number of bacteria was obtained. Sterilizing the lobe of the ear as well as possible with green soap and alcohol, and then collecting the blood in a pipette directly from the puncture and not by dropping it into a tube, reduced greatly contamination with skin staphylococci. Over 75 per cent of the plates thus prepared gave no staphylococcus colonies, but pure cultures of the infecting organism.

Phagocytic experiments.—The usual technic was followed. Smears were made at the end of 15 or 20 minutes and also at the end of 1 hour and of 12 to 24 hours. The average number of bacteria taken up per leukocyte was obtained by counting the number in at least 50 leukocytes and frequently in 200 leukocytes. The percentage of leukocytes engaged in phagocytosis corresponded closely with the bacterial counts. The suspensions were made in NaCl solution from agar-grown pneumococci as well as of 24-hour cultures in dextrose broth. The greatest care was exercised to make the results comparable. The normal blood used for the controls was obtained at the same time as the patient's, the washing was done in exactly the same manner with three changes of 50 times the quantity of salt solution. The number of leukocytes in the washed

blood were controlled by actual counts; differential counts were made to establish that not only the total number of whites but that the number of polymorphonuclears were comparable. The suspensions from day to day were made as nearly alike in density as possible. Carbol thionin chiefly was used, altho frequent controls with Giemsa's and Leishman's stains were made.

Pneumococcidal tests.—The mixtures (see Tables 1 and 2) were the same as used to determine phagocytosis, except that the suspensions of pneumococci were diluted 40 to 50 times. Equal quantities of washed blood, serum, and diluted suspension were drawn into the capillary end of glass tubes and thoroughly mixt. A small loop was plated in blood agar immediately and at the end of 24 hours, at which time repeated trials showed conclusive results are best obtained. The loop contained approximately $\frac{1}{20}$ to $\frac{1}{30}$ of the total mixture. Control tests with larger quantities in test tubes gave similar results. The uniformity in the number of colonies which developed in the plates made immediately show the method is a thoroughly reliable one. Sterile plates, however, are not obtained so often as when larger quantities of blood are used in test tubes. This is no doubt due to the larger surface covered at the sides of the pipette in the mixing process where drying usually prevents the complete destruction of the bacteria by the leukocytes. Smears at the end of 18 to 24 hours of both bacteriolytic and phagocytic mixtures were studied as a means of control of the plate method. Here the erythrocytes serve as a means of estimating the number of bacteria in comparable mixtures.

INTRALEUKOCYTIC DESTRUCTION OF PNEUMOCOCCI FROM ENDOCARDITIS.

It has been shown that the pneumococcidal action of normal and pneumonic blood is due to intraleukocytic destruction of pneumococci.[1] The pneumococci used in my experiments were isolated from the blood and sputum of patients with pneumonia and had become non-virulent by artificial cultivation, and hence were freely susceptible to phagocytosis. In order to study whether the destructive action of blood on endocarditis cocci also is due to the same cause, a series of experiments, of which Table 1 is illustrative, were made.

The upper layer of thoroughly washed blood was decanted and used for leukocytic mixtures while the botton layer containing few leukocytes was used for non-leukocytic mixtures. The experiment in Table 1 was made February 25, after 18 injections of normal serum were given intravenously in Case 362. The destructive power at this time was equal to that of normal blood as shown in Experiments 1, 2, 3, and 4, Table 1. The pneumococcidal power here, as for other pneumococci, is proportionate to the number of leukocytes

[1] Rosenow, *Jour. Infect. Dis.*, 1906, 3, p. 683.

present and to the degree of phagocytosis. Destruction is absent where phagocytosis is prevented by taking away the leukocytes; by addition of broth (Experiments 5 and 6); by heating the serum (Experiments 7 and 8); and by using a highly virulent pneumococcus which is insusceptible to phagocytosis (Experiments 11 and 12). Crucial evidence of the effect of phagocytosis on the growth of pneumococci is afforded in Experiments 7, 8, 9, and 10: It has been found repeatedly that the pneumococcus grows equally well in heated and

TABLE 1.

The Destructive Effect of Leukocytes on Endocarditis Pneumococci.

Each Mixture Contains Equal Parts of Washed Blood, Serum, or Broth Pneumococcal Suspension	Phagocytosis (15 Min.)	Colonies on Blood Agar Plates			
		32–34,000 Leukocytes per c.c.		10 Leukocytes per c.c.	
		Immed.	24 Hrs.	Immed.	24 Hrs.
1. Normal blood + normal serum + pneumococ. 362	1.9	2	200	1	950
2. Normal blood + serum 362 + pneumococ. 362	2.3	5	50	2	600
3. Blood 362 + normal serum + pneumococ. 362	3.5	5	0	2	1,200
4. Blood 362 + serum 362 + pneumococ. 362*	2.6	2	8	1	750
5. Normal blood + broth + pneumococ. 362	0.4	0	5,000	10	5,000
6. Blood 362 + broth + pneumococ. 362	0.3	0	4,600	2	4,350
7. Normal blood + heated normal serum + pneumococ. 362	0.2	4	1,000		
8. Blood 362 + heated serum 362 + pneumococ. 362	0.3	25	2,000		
9. Normal blood + normal serum (heated 10 parts, unheated 1 part) + pneumococ. 362	1.5	6	150		
10. Blood 362 + serum 362 (heated 10 parts, unheated 1 part) + pneumococ. 362	2.8	5	14		
11. Normal blood + normal serum + pneumococ. 356†	0.3	6	3,200	5	3,750
12. Blood 362 + serum 362 + pneumococ. 356	0.3	8	4,500	3	5,400

* This experiment was performed when both phagocytosis and bacteriolysis was nearly normal to the homologous strain.

† Pneumococcus 356 highly virulent.

unheated homologous serum, hence the difference obtained in Experiments 7 and 8 where heated normal serum and heated patient's serum are used, and Experiments 9 and 10, in which one part of unheated serum is added to ten parts of heated serum, cannot be due to differences in rapidity of growth of the organism, but must be due to the activation of the serum with resultant increased phagocytosis and intraleukocytic destruction.

In the experiments bearing on Case 408 also, intraleukocytic destruction took place freely and was readily prevented by reducing or preventing phagocytosis by using non-leukocytic mixtures, by using NaCl solution instead of serum, by substituting heated for unheated

serum, by cultivating the cocci in the serum alone without leukocytes, by using highly virulent pneumococci, and by rendering the homologous cocci insusceptible to phagocytosis by previously growing them in fresh normal and patient's serum.

In certain instances (Tables 2 and 3) when the blood from cases of endocarditis appear to lack destructive power it was restored by

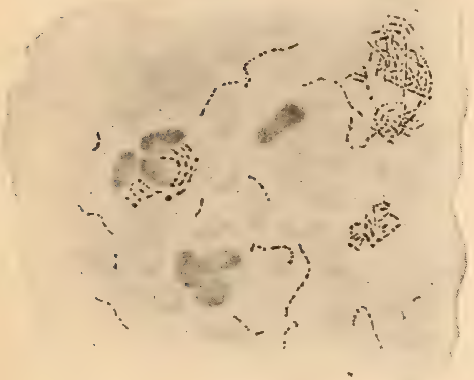

Fig. 1.—Smear made at end of 24 hours of a phagocytic mixture containing equal parts of washed 362 blood, 362 serum, and of pneumococcus 362 suspended in NaCl so u-tion after cultivation on agar. ×1.000.

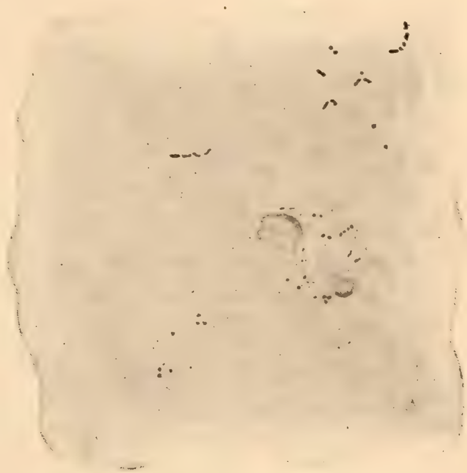

Fig. 2.—The same as (1) except that the mixture contains washed normal blood and normal serum instead of patient's. ×1,000. Note the marked growth, the long chains, the clumps, and the phagocytosis and absence of intraleukocytic destruction of pneumococci in Fig. 1, the leukocytes being well preserved. Fig. 2 shows marked phagocytosis and intraleukocytic destruction of pneumococci with only a few free pneumococci and disintegrating leukocytes.

the addition of varying amounts of normal serum. In mixtures of the same amounts of normal serum and of the patient's serum, but without leukocytes, there would be no destruction; hence the result obtained clearly is not due to the activation of free lysins. This is what would be expected because the organisms grow freely in normal serum and because of the distinct morphologic evidences of intraleukocytic destruction in active mixtures. The study of smears at the end of 18 and 24 hours' incubation (Tables 1 and 2) showed the polymorphonuclear leukocytes to present marked evidence of disintegration in the mixtures in which there was destruction of pneu-

mococci. This disintegration of leukocytes was always most marked where the evidence of intraleukocytic destruction of pneumococci was greatest. The leukocytes in the mixtures where destruction was absent, even though phagocytosis might be marked, and those not engaged in phagocytosis at all, whether in active or inactive mixtures, were always better preserved. Hence the destruction of ingested pneumococci by the leukocytes seems to cost their life.[1]

The activity of the patient's leukocytes as compared with normal leukocytes.—Wright and his followers advanced the view that the source of the leukocytes in opsonic estimations is a matter of indifference, but recent investigations indicate that this view is no longer tenable.[2]

In my own work[3] distinct differences were found in the phagocytic activity of the leukocytes in certain infections as compared with normal, and especially when the phagocytic power of the patient's leukocytes and serum was compared with the phagocytic power of normal leukocytes and serum—opsono-phagocytic index of Glenn and Cox.[4]

Tables 2, 3, and 4 illustrate the results obtained in the series of cases now considered. They show that changes in the leukocytes may play an important part in the phagocytic, and especially the destructive power of the blood.

Table 2 shows that the lack of destruction of pneumococci (Experiments 2 and 4) cannot be due to absence of phagocytosis,[5] because

[1] In this connection the recent work of Werbitzki (*Arch. f. Hyg.*, 1909, 70, p. 270) and Zeissler, (*Mitteil. a. d. Hamburgischen Staatskrankenaustalten*, 1909, 9, p. 167) who studied the destructive power of leukocytes over various pathogenic bacteria, must be mentioned. The former used human serum and leukocytes (obtained from the blood); the latter, rabbits' serum and leukocytes (obtained from the pleural cavity). The number of the leukocytes used in the mixtures was not accurately determined. They concluded that serum and leukocytes have no more destructive power than serum alone.

An analysis of their own tables show that in most instances where the number of bacteria is not too overwhelmingly large the mixtures which contain serum and leukocytes actually show a relatively greater destruction than in those containing serum alone. This is particularly true with respect to staphylococci, streptococci, and pneumococci. Moreover, they take no recognition of the fact that the presence of red blood corpuscles (hemoglobin) in serum makes it a better culture medium especially for pneumococci and streptococci.

In order to secure sterile plates, in a serum-containing mixture, it is necessary to obtain a correct balance between the number of leukocytes and bacteria on the one hand and their susceptibility to phagocytosis on the other. Two factors are involved—growth of bacteria which are free in the serum, and phagocytosis. If the former gets the over hand the leukocytes become packed with bacteria and no evidence of intra-leukocytic destruction can be made out.

[2] T. H. Boughton, *Jour. Infect. Dis.*, 1910, 7, p. 111. Here are given references to the literature.

[3] *Jour. Infect. Dis.*, 1906, 3, p. 683; *ibid.*, 1909, 6, pp. 245 and 296.

[4] *Jour. Path. and Bact.*, 1909, 14, p. 90.

[5] Phagocytosis here is used to designate the mere taking up of the bacteria by leukocytes.

the phagocytosis is alike in the mixtures which show marked destruction of pneumococci and those which show marked growth. The fault seems to be a deficiency of something in the serum which has to do with intraleukocytic destruction quite independently of opsonin.

The experiment described in Table 2 illustrates daily experiments from January 10 to March 4 in Case 262 (Chart 1).

TABLE 2.

Phagocytosis and Destruction of Pneumococcus 362 by Normal and by Patient's (362) Leukocytes in Normal and Patient's Serum.

Mixtures Each mixture contains equal parts of washed blood (20,000 leukocytes per c.mm.), serum, and suspension of pneumococcus 362	Phagocytosis (15 Min.)	Smears after 24 Hours at 37° C.	Colonies on Blood Agar Plates	
			Immed.	24 Hrs.
1. Normal leukocytes and normal serum	1.6	Phagocytosis moderate; decided evidence of digestion of pneumococci; some leukocytes show marked disintegration, others intact	30	14
2. Normal leukocytes and serum 362	1.1	Phagocytosis marked; no intraleukocytic digestion; many diplococci in long chains and clumps; leukocytes well preserved	35	2,500
3. Leukocytes 362 and normal serum	1.2	Phagocytosis slight; marked evidence of intraleukocytic destruction of pneumococci; some leukocytes which show destruction of pneumococci markedly disintegrated; the others stain well; few free pneumococci	28	15
4. Leukocytes 362 and serum 362	1.4	Phagocytosis marked; no intraleukocytic digestion; many diplococci in long chains and bunches; leukocytes well preserved	22	1,550
5. Leukocytes 362, normal serum, and serum 362, equal parts	1.9	Phagocytosis slight; intraleukocytic digestion decided; few diplococci; leukocytes not well preserved	20	40
6. Leukocytes 362 and serum (normal 1 part and serum 362, 6 parts)	1.2	Phagocytosis moderate; marked evidence of intraleukocytic destruction; few free pneumococci; leukocytes showing the greatest destruction of pneumococci are proportionately disintegrated	26	150
7. Leukocytes 362, and serum (normal 1 part and serum 362, 12 parts)	1.6		23	25
8. Leukocytes 362 and serum (normal 1 part and serum 362, 48 parts)	1.7	More growth than in experiments 2 and 5; chains are shorter; more diplococci	47	370

The experiment described in Table 3 illustrates frequent experiments from June 10 to August 7 in Case 408 (Chart 2).

In Table 3 the lack of destruction is confined to the mixture in which the patient's serum and leukocytes were combined (Experiment 4) and appears to be due to diminished phagocytosis. Phagocytosis and intraleukocytic destruction are up to the normal in the mixtures containing the patient's serum and normal leukocytes and the patient's leukocytes and normal serum (Experiments 2 and 3).

On what does the peculiar behavior toward the infecting bacteria of the patient's blood, as illustrated in Tables 1 and 2, and by the curves in Charts 1 and 2, depend? In Case 362 (Table 2) the fault seems to lie with the serum alone, because normal and patient's leukocytes behave alike in the patient's serum. The serum in this instance, while it has practically a normal amount of opsonin, is deficient in something which is necessary to destroy the ingested cocci. Now this lack of destructive power, even tho phagocytosis was up to the normal standard, was always specific for the infecting pneumococcus.

TABLE 3.

PHAGOCYTOSIS AND DESTRUCTION OF PNEUMOCOCCUS 408 BY NORMAL AND BY PATIENT'S (408) LEUKOCYTES IN NORMAL AND IN PATIENT'S SERUM.

MIXTURES Each mixture contains equal parts of washed blood (25,000 leukocytes per c.mm.), serum, and suspension of pneumococcus 362	PHAGOCYTOSIS (20 MIN.)	SMEARS AFTER 24 HOURS AT 37° C.	COLONIES ON BLOOD AGAR PLATES	
			Immed.	24 Hrs.
1. Normal leukocytes and normal serum	2.58	Phagocytosis marked; digestion of bacteria and leukocytic disintegration; few bacteria, mostly diplococci	2,300	175
2. Normal leukocytes and serum 408	2.2	Same as above, but long chains and clumps	2,150	250
3. Leukocytes 408 and normal serum	2.66	Leukocytes better preserved	2,675	189
4. Leukocytes 408 and serum 408	0.7	Phagocytosis marked; digestion absent; leukocytes well preserved; marked growth; long chains and clumps	2,200	4,300
Leukocytes 408+				
5. Serum (normal serum 1 part, serum 408, 1 part)	2.7		2,000	75
6. Serum (normal serum 1 part, serum 408, 5 parts)	2.4	Phagocytosis decided; digestion of bacteria present; leukocytes only fairly well preserved; agglutination; few bacteria	2,350	125
7. Serum (normal serum 1 part, serum 408, 10 parts)	2.0		1,890	60
8. Serum (normal serum 1 part, serum 408, 50 parts)	2.9		2,750	120

In Table 3, on the other hand, where both phagocytosis and destruction are below normal, it seems to be a fault of the patient's leukocytes when in their own serum, because they are as active as normal leukocytes in normal serum.

Experiments with other strains of pneumococcus, with streptococcus, and with staphylococcus show that in mixtures of homologous serum and leukocytes, low phagocytosis was largely, but not always, peculiar to the infecting pneumococcus.

The study of phagocytosis and intraleukocytic destruction of pneumococci from day to day in these cases shows that the two

processes, while they usually run hand in hand, seem to be independent. Whenever phagocytosis was low in the mixtures containing patient's serum or leukocytes, destruction was correspondingly slight also, but on the other hand when phagocytosis was well up to the normal or above, destruction was usually, but not always, correspondingly marked. Phagocytosis by patient's leukocytes as well as by normal leukocytes might be more marked than in normal blood, yet intraleukocytic destruction in the patient's serum might be entirely absent. The addition of normal serum to the patient's serum in these cases always brought the phagocytosis and destruction up to the normal where either or both were deficient (see Tables 2 and 3), but at no time did it raise the phagocytic or destructive power to a point above that of normal blood.

It was also found that the phagocytic activity of the leukocytes of endocarditis patient 408 varied greatly from day to day, especially when tested in the patient's own serum. In 81 tests the phagocytic activity of the patient's leukocytes with normal serum—cytophagic index of Glenn and Cox—was found greater than that of normal leukocytes with normal serum 51 times, less 28 times. The phagocytic activity of the patient's leukocytes when suspended in the patient's serum—opsono-cytophagic index of Glenn and Cox—on the other hand, was greater than that of normal leukocytes in normal serum only 32 times, and less 47 times—practically the reverse of the first.

The opsonic curves in Charts 1 and 2 show that with few exceptions the days on which the opsonic index of the patient's serum is high or low, the activity of the patient's leukocytes in normal serum is correspondingly high or low; and the dates on which the opsonic power of the patient's serum is high or low, as measured with the patient's leukocytes, the activity of the patient's leukocytes in the patient's serum is correspondingly high or low. The phagocytic activity of the patient's leukocytes in normal serum shows less variation from day to day than when they are suspended in the patient's serum. In the patient's serum (Table 2) there is often a drop in phagocytosis, as if the serum had an inhibitory action on the leukocytes. When leukocytes show a lack of activity in their own serum their activity often is normal or greater than normal in normal serum,

as if the normal serum contained something that activated the patient's leukocytes.

In three experiments the phagocytic as well as the destructive power of the patient's leukocytes was brought up to normal by suspending them (after washing) in normal serum for one and one-half hours, then washing again, and adding cocci previously opsonized (Tables 4 and 5).

If the diminished phagocytosis in the patient's serum was due to lack of opsonin and the serum had no inhibitory action on the leukocytes, then the phagocytic activity ought to be the same as that of normal leukocytes with respect to previously opsonized pneumococci (the cocci being washed after treatment with serum). The fact is that in six tests the activity was found to be less than normal in the presence of serum and in six greater than normal when opsonized ("sensitized") cocci were used. In other words, the patient's serum seems to have had a definite inhibitory action on the leukocytes. The phagocytic activity of the patient's leukocytes in normal serum and toward pneumococci previously sensitized in normal serum corresponded closely on these dates, which again indicates that the patient's serum exerted an inhibitory effect on its leukocytes. Another evidence that the diminution in phagocytosis in mixtures containing homologous serum, leukocytes, and pneumococci frequently observed in these cases, cannot be due to the failure of opsonification, but must be due to an inhibitory action of the serum on the leukocytes, is seen in the fact that pneumococci on suspension in the patient's serum remove its opsonin to the same extent as they do that of normal serum.

The curves (Charts 1 and 2) show that the cytophagic index of the patients was often pronounced during the leukocytosis which followed the occurrence of joint involvement, petechiae, and embolic processes, during the natural course of the disease, as well as after the injection of dead cocci or serum or both; finally, and this is more important, the destructive power of the patient's leukocytes in the patient's serum following these reactions was usually up to the normal or above, while frequently greatly reduced or absent previously.

The relationship between leukocytes, serum, and cocci was analyzed more closely by using cocci that had been treated with

serum, or "sensitized," which means that the cocci were suspended in serum at 37° C. for one and one-half hours and then washed once in salt solution. The cocci used in the experiments given in Tables 4 and 5 were sensitized in exactly the same way and suspended after washing in equal amounts of NaCl solution. The suspensions were rather thin, which explains the small amount of phagocytosis in the mixtures containing pneumococcus 408. This strain had been cultivated on agar for only 48 hours previously and was not as freely taken up as pneumococcus 409, which had been grown for 30 days on blood agar. The suspensions of pneumococci for the study of destruction were made by diluting those for the phagocytic tests 40 times.

Table 4 shows that the destruction of pneumococci is closely dependent on the degree of phagocytosis. The destructive action of the patient's leukocytes (Case 408) is more marked than that of normal leukocytes with respect to homologous cocci sensitized in normal serum and less so in case the cocci are sensitized in the patient's serum. Toward strain 409 the leukocytes of Case 408 have greater destructive action, but here it makes no difference whether the cocci are sensitized in normal or in the patient's serum.

TABLE 4.

Phagocytosis and Intraleukocytic Destruction of "Sensitized" Pneumococcus 408 and 409 in the Absence of Serum.

Mixtures Equal parts of suspension of washed blood (15,000 leukocytes per c.mm.) and suspension of sensitized pneumococci		Phagocytosis (20 Min.)		Colonies on Blood Agar Plate			
				Immediately		24 Hours	
		408	409	408	409	408	409
Cocci sensitized in normal serum +	Normal leukocytes..	0.1	1.38	350	1,200	1,570	2,400
	408 leukocytes	0.2	1.42	320	1,650	750	0
Cocci sensitized in 408 serum +	Normal leukocytes..	0.1	3.1	1,250	625	3,200	1,200
	408 leukocytes......	0.32	2.3	780	700	4,300	0

This experiment was made July 7 when the opsonic power of the patient's serum (in the presence of homologous leukocytes) was well up to the normal. The experiment in Table 5 was made two days later, when the opsonic power of the patient's serum had fallen somewhat. Now the phagocytic activity of the patient's leukocytes is greater than that of normal leukocytes whether the cocci are sensitized

in the unheated and heated normal or patient's serum, but there is now no destructive power on the part of the patient's leukocytes with respect to cocci sensitized either in normal or in patient's serum. In the presence of normal serum, however, the same leukocytes digested cocci as well as normal leukocytes. In other words it seems that during these two days the leukocytes have been so modified that their power to destroy the ingested cocci in the absence of serum is wholly lost. The fault here must be due to changes in the leukocyte and not in the serum alone, because normal leukocytes digest the cocci sensitized both in the patient's and in normal serum.

TABLE 5.

Phagocytosis and Intraleukocytic Destruction of Sensitized Pneumococcus 408 in the Absence of Serum.

Mixtures Equal parts of suspension of washed blood (14,000 leukocytes per c.mm.), and suspension of sensitized pneumococci		Phagocytosis (20 Min.)	Colonies on Blood Agar Plates	
			Immediately	24 Hours
Cocci sensitized in unheated normal serum +	Normal leukocytes..	1.6	500	350
	408 leukocytes......	2.6	850	4,500
Cocci sensitized in heated* normal serum +	Normal leukocytes..	0.26	450	3,200
	408 leukocytes......	0.3	650	4,500
Cocci sensitized in unheated 408 serum +	Normal leukocytes..	1.06	540	450
	408 leukocytes	2.02	450	3,500
Cocci sensitized in heated 408 serum +	Normal leukocytes..	0.1	375	3,280
	408 leukocytes	0.5	650	4,800

* Heated = 60° C. for 1 hour.

In view of the possible objection that the smears at the end of 20 minutes are not an accurate measure of phagocytosis, it should be stated that smears were made of the phagocytic mixtures at the end of one hour and of 18 and 24 hours. The phagocytosis per leukocyte at the end of one hour, while greater, corresponds closely to that at the end of 20 minutes. The evidence of intraleukocytic destruction of pneumococci in the smears at the end of 18 to 24 hours was greater in the mixtures in which the plate method showed the greatest diminution in the number of viable cocci. Experiments like these were made on seven other dates with similar results. In further study of the mode of destruction, washed non-leukocytic blood was used side by side with washed leukocytic blood. Here the growth of cocci previously treated in normal or in patient's serum was equally rapid. Phagocytability for normal leukocytes of agar-grown pneumococci treated in normal serum was always found to

run hand in hand with intraleukocytic destructibility. The patient's cocci treated in the patient's serum, on the other hand, may be taken up freely, but they would not be digested by the patient's leukocytes. In other words, phagocytability and destructibility of the cocci previously treated in the patient's serum, while usually running parallel, do not always do so, as in the mixtures containing serum. The lack of destructive power by the patient's leukocytes was found to be specifically related to the homologous cocci previously treated in the patient's serum.

The phagocytic activity of the patient's leukocytes, determined in this way, was greater than normal leukocytes in all the tests made with the cocci previously treated in normal serum and in all but two instances when they had been previously treated in the patient's serum. The increase in the activity of the leukocytes corresponded to the leukocytosis in the patient.

Pneumococci previously treated in serum and than washed are not only rendered phagocytable, but digestible by normal leukocytes. Other things being equal, the number of leukocytes must be larger than where the serum is not removed in order to obtain sterile plates as would be expected, because pneumococci grow quite as readily in washed blood as in the mixtures where serum is present, but the young cocci, not being opsonized, are not taken up and hence the total growth is more marked. The reason sterile plates were not obtained in Tables 4 and 5 is no doubt due to this cause.

The facts obtained in this way regarding the peculiar relationship between homologous leukocytes, serum, and cocci indicate that the serum may affect the leukocyte so as to render it relatively inactive, even to such an extent that it no longer digests cocci sensitized in normal serum. But that the pneumococcus also is responsible to some degree is shown by the resistance of the infecting strain to destruction, whether in the presence of serum or after sensitization.

PHAGOCYTOSIS AND INTRALEUKOCYTIC DESTRUCTION OF ENDOCARDITIS PNEUMOCOCCI AFTER PROLONGED CULTIVATION IN SERUM.

I have shown that cocci from cases of endocarditis become resistant to phagocytosis when cultivated in normal serum, but remain susceptible to phagocytosis when grown in heated serum. The organ-

isms obtained from the blood during life also resist phagocytosis. The following experiments were made to study the effect of the continuous cultivation of pneumococci from endocarditis in normal serum and in the patient's serum on their susceptibility to phagocytosis and their virulence on the one hand, and on the ease with which they are destroyed within leukocytes on the other.

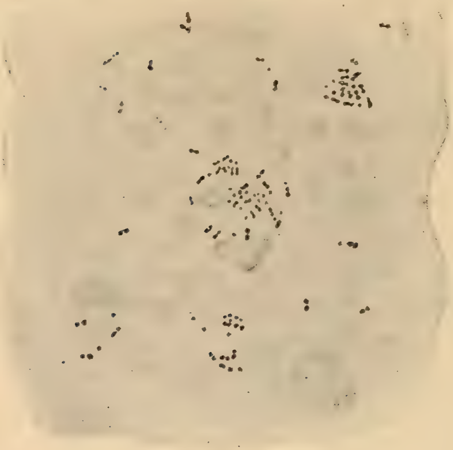

Fig. 3.—Smears made at the end of 24 hours of mixtures of equal parts of washed 408 blood, 408 serum, and suspension of pneumococcus 408 in NaCl. Intraleukocytic destruction of pneumococci and a corresponding leukocytic disintegration. ×1,000.

Fig. 4.—The same strain as in Fig. 3 grown in the patient's blood. Note the long chains and the entire absence of phagocytosis. The leukocytes are preserved perfectly. ×1,000.

Beginning July 7, normal and patient's blood (Case 408), obtained on the dates recorded on Chart 2, were used while fresh, as culture media for a strain of the pneumococcus (408), which at no time had been cultivated on artificial media. The amount of phagocytosis and intraleukocytic digestion in these cultures were studied from day to day by making smears from the layer joining corpuscles and serum at the end of 24 and 48 hours. Practically no phagocytosis could be found in the early part of each experiment in either normal or in patient's blood (see Fig. 2); later the cocci became somewhat more susceptible to phagocytosis. This lack of phagocytosis was not due to death of the leukocytes because tests showed that the leukocytes were still active at the end of the experiment. Moreover,

cultivation in washed blood and heated serum showed an enormous apparent phagocytosis at the end of 24 hours. That the acquired resistance to phagocytosis of these bacteria is dependent on substances in the serum which deteriorate rapidly, is indicated also by the fact that phagocytosis was always more marked in the subcultures made after the organisms had been cultivated in the blood without transfer for 48 and especially 72 hours than when cultivated for only 24 hours.

The marked degree of phagocytosis at the end of 24 hours of the organisms cultivated in heated serum to which washed leukocytes are added might be due to phagocytic ingestion of all the cocci or the result of growth within the leukocytes of a relatively small number taken up in the early part of the experiment.

It is also possible that cultivation in serum of the bacteria may render them more resistant to intraleukocytic destruction. A careful study of smears of cultures in normal and in patient's blood at the end of 24 hours on the dates indicated in Chart 2 seems to show that the relatively few organisms which were taken up acquired a resistance to intraleukocytic destruction. See the experiments given in Table 6.

The washed cocci after having been cultivated in serum 408 for 30 days appear highly resistant to phagocytosis in unheated normal and in patient's serum, but apparently freely phagocytable in heated serum. In the second part of the table, in which agar-grown cocci are used, phagocytosis is pronounced in the presence of unheated serum and practically absent in heated serum.

The smears from mixtures containing the washed serum-grown cocci at the end of 24 hours fail to show any evidence of digestion of the relatively few cocci taken up in the presence of unheated serum. The agar-grown cocci, on the other hand, show relatively marked destruction. The cocci which were grown in serum are freely taken up when suspended in heated serum, and show some definite evidence of intraleukocytic destruction by normal leukocytes in normal and in patient's serum, but not by patient's leukocytes in patient's serum. In none of these mixtures, however, was the digestion nearly as marked as in those containing agar-grown cocci in unheated serum. The few agar-grown cocci which were taken up in heated serum show

no evidence of digestion whatsoever. The inability to digest serum-grown cocci was found in the case of those cultivated in normal and as well of those grown in patient's serum. Plates made immediately and at the end of 24 hours showed the growth to be correspondingly

TABLE 6.

PHAGOCYTOSIS AND INTRALEUKOCYTIC DESTRUCTION OF PNEUMOCOCCUS 408 GROWN IN 408 SERUM AND ON AGAR.

MIXTURES	UNHEATED SERUM		SERUM HEATED AT 60° C. 1 HOUR	
Each mixture contains equal parts of washed blood (18,000 leukocytes per c.mm.), serum, and suspension of pneumococcus 408	Phagocytosis (20 Min.)	Smears after 24 Hours at 37° C.	Phagocytosis (20 Min.)	Smears after 24 Hours at 37° C.
Pneumococcus 408 grown in serum 408 and washed in NaCl solution:				
Normal leukocytes and normal serum	0.20	Decided growth; short chains and diplococci; phagocytosis slight; no digestion	6.40	Marked growth; phagocytosis extremely marked; leukocytes packed with pneumococci; some digestion
Leukocytes 408 and normal serum	0.14		5.30	
Normal leukocytes and 408 serum	0.32	Marked growth; long chains and clumps; little phagocytosis; no digestion	2.30	Marked growth; phagocytosis less than above, but greater than in unheated serum; leukocytes disintegrated; digestion present, but less than where agar-grown cocci are used
Leukocytes 408 and 408 serum	0.08		2.20	Growth marked; phagocytosis slight; no digestion; leukocytes well preserved
Pneumococcus 408 grown on agar:				
Normal leukocytes and normal serum	4.16	Slight growth; few diplococci; phagocytosis slight but digestion marked; leukocytes disintegrated	0.46	Growth marked; diplococci; short chains; phagocytosis decided; no digestion; leukocytes well preserved
Leukocytes 408 and normal serum	3.48		0.46	
Normal leukocytes and 408 serum	1.44	More growth; long chains; phagocytosis decided; digestion present; leukocytes disintegrated	0.36	As above, but longer chains
Leukocytes 408 and 408 serum	1.98	Growth extremely marked; long chains and clumps; phagocytosis marked; digestion slight; leukocytes fairly well preserved	0.66	

greater in the mixtures where no evidences of digestion could be made out even tho phagocytosis is quite considerable. The leukocytes which show phagocytosis but show no digestion and those which contain no pneumococci are well preserved at the end of 24 hours, while those which show evidence of digestion of serum-grown cocci

are disintegrated just as in the mixtures containing agar-grown cocci. The cocci grown in normal serum for 30 days were more susceptible to phagocytosis than those grown in the patient's serum. The agar-grown cocci acquire resistance to phagocytosis in normal serum provided a relatively small number are inoculated; if a large number are introduced they remain freely susceptible to phagocytosis, probably because opsonin is absorbed at the same time as the cocci multiply.

The diminished phagocytosis in Table 7 both by normal and by patient's blood of the organisms previously grown in normal and in patient's blood must be due to relative resistance to phagocytosis; one single subculture in ascites broth, however, rendered the cocci quite freely susceptible. Smears at the end of 24 hours now show distinct evidence of intraleukocytic destruction.

TABLE 7.

Relative Resistance to Phagocytosis of Pneumococci Grown on Agar and on Serum.

Pneumococci (No. of pneumococci the same in each mixture.)	Phagocytosis (20 Min.)	
	Washed Normal Blood and Normal Serum	Washed 408 Blood and 408 Serum
Pneumococcus 408 cultivated on agar for 4 weeks and in ascites broth for 24 hours	9.8	3.28
Pneumococcus 408 cultivated in normal blood for 4 weeks and in ascites broth for 12 hours	5.6	1.86
Pneumococcus 408 cultivated in 408 blood for 4 weeks and in ascites broth for 24 hours	3.84	1.12

In the case of pneumococcus 409 similar tests were made by cultivating the patient's strain directly from the blood in the serum obtained after death by transfer every other day for two weeks. At first the cocci resisted phagocytosis completely, but later, as the serum grew old, they gradually became as freely taken up as they were in heated serum in the beginning. Conclusive evidence of intraleukocytic digestion could not be made out.

From observations like those given in Table 6 it is clear that the cocci absorb opsonin freely when grown in fresh normal and patient's serum, because they become susceptible to phagocytosis by washed leukocytes in heated serum, but when thus saturated with opsonin they are insusceptible to phagocytosis in normal and patient's unheated serum. An exact explanation of this peculiar phenomenon cannot be offered. Perhaps the concentration of absorbed opsonin within the

cocci and free opsonin in the serum plays a rôle, because repeated washing of the cocci make them more susceptible to phagocytosis in unheated serum and because those washed only once are taken up in diluted serum much as they are in the heated serum.

The resistance to phagocytosis of serum-grown cocci in unheated serum is somewhat analogous to the unexplained failure of agglutination of certain strains of typhoid bacilli and other bacteria to take place in low dilutions of serum while it occurs freely in high dilutions.

SUMMARY.

A study from day to day of the number of bacteria in the blood, the opsonic power of the serum, the phagocytic and the destructive power of the blood in chronic endocarditis, brings out the interesting facts that the number of bacteria increases and the destructive power of the blood decreases for a variable time previous to the occurrence of embolism and joint infections (see Charts 1 and 2). The opsono-cytophagic index of the patient at this time usually appears to be high. During the reaction after embolism and arthritis, the peculiar behavior of the patient's serum and of the leukocytes disappears, the destructive power of the blood returns, the number of bacteria in the blood shows a corresponding drop, the patient feels better but is weaker than before. Hence the occurrence of embolism and of other intercurrent processes in endocarditis is associated with a definite lowering of the destructive power of the patient's blood.

Phagocytosis and intraleukocytic destruction of endocarditis pneumococci by normal and by patient's leukocytes in normal serum always run hand in hand. Phagocytosis and intraleukocytic destruction by patient's and by normal leukocytes in patient's serum, on the other hand, do not always run parallel. While lowered phagocytosis was always associated with lowered destruction, normal and even increased phagocytosis was frequently associated with complete absence of intraleukocytic destruction and consequent marked growth of pneumococci. Hence phagocytosis and intraleukocytic destruction, while closely related, are independent.

In the instances when no destruction occurs, the patient's serum either is deficient in something necessary to make the cocci digestible or it contains a substance which alters the leukocytes so that they are

unable to destroy the ingested cocci. Certain experiments with sensitized cocci and in absence of serum clear up this point: Normal leukocytes digest the cocci sensitized in the patient's serum quite as readily as those sensitized in normal serum, hence the inability of normal leukocytes to digest the pneumococci in the actual presence of the patient's serum must be due to an effect on the leukocytes by the serum. The patient's leukocytes may be unable to digest the cocci sensitized in patient's serum, even tho more active than normal phagocytically, and yet digest fairly well those sensitized in normal serum; and at another time they may be unable to digest the cocci whether sensitized in normal or patient's serum. This was found to be specific for the infecting strain of pneumococcus. There seems then to be a substance in normal leukocytes, which at times is lacking in the patient's leukocytes and which has to do with intra-leukocytic digestion and is independent of opsonin—a substance which is present in normal serum because patient's leukocytes take it up from normal serum. Normal leukocytes in patient's serum at times digest pneumococci when patient's leukocytes do not. More often, however, normal leukocytes in the patient's serum lose the power to digest the cocci, even tho phagocytosis is normal, while they at the same time digest cocci previously sensitized in the patient's serum and then washed—good evidence that the serum of the patient at times is not only lacking in the substance necessary for digestion, but that it contains something which neutralizes that carried by washed leukocytes and present in small quantities of normal serum. This affords an explanation why smaller quantities than one part of normal to forty parts of patient's serum failed to activate patient's blood *in vitro.* The way normal serum activates the patient's blood then must be by neutralizing this substance and supplying an excess of it which the leukocytes need. In other words, it seems that normal serum has in it a substance which acts on the leukocytes which is independent of opsonin, and which has to do especially with intra-leukocytic digestion. The patient's serum at the time when the destructive power is absent is not only deficient in this substance, but frequently contains an antagonistic body.

A leukocyte count of 10,000 would give 10,000,000 leukocytes per c.c. to take up, let us say, 100 cocci per c.c., which is a fair average of

the number present. This makes 100,000 leukocytes per pneumococcus. The fact that the pneumococci are not destroyed under such circumstances would seem to be good proof that the mechanism by which the cocci protect themselves is a reliable one.

The question whether the cocci actually multiply in the blood in cases like those under consideration has been much discussed. It is usually assumed that they do, but no direct evidence to prove this point has been brought out in the past. The results obtained by cultivating the cocci in defibrinated blood would make one think growth takes place in the blood *in vivo*. Definite evidence has come to light in the study of these cases that in cultures from the blood we are dealing with cocci which have been washed into the circulation from the thrombotic growths on the valves as well as those which have actually multiplied within the blood. The latter differ from the former in that they resist phagocytosis and fail to ferment inulin; the former are freely susceptible and ferment inulin. Similar results were reported in my previous paper. Two factors then seem to be responsible for the continuation of the infection—the focus on the heart valve acting as a feeder, and the multiplication of the cocci within the circulation. The former would seem to resist healing from the protection afforded the cocci by the large thrombotic masses on the valves; the latter by a process of immunization against the antibodies of the host. In acquiring resistance to phagocytosis and what is more important, resistance to intraleukocytic digestion, the cocci also probably produce changes in the serum which alters the leukocytes so that they become less active phagocytically in the patient's serum and less able to digest the infecting cocci.

The therapeutic injection of killed pneumococci in this class of cases holds out little hope in the way of cure. Small doses sometimes seem to produce temporary improvement. Large doses certainly do harm. The injection of normal serum seems to have a decided influence in changing the peculiar conditions of the blood. In cases 362 and 408 the injections were associated with a definite reaction and temporary improvement. The sharp reactions obtained in the beginning from intravenous injections of small doses would seem to indicate that transfusion of blood in these cases might be dangerous.

Horder failed to obtain an immune serum by repeated injections

with cocci from cases of endocarditis of animals. The results of the animal experiments make the outlook for a specific serum therapy gloomy. The more one learns of the mechanism of the infection in these cases the more difficult the discovery of a specific therapy would seem to be.

Finally, in the light of my results there is still another point which should be emphasized. Practically all of the cases of endocarditis give a definite history of a previous endocarditis which has healed, leaving injured and deformed valves. The first attack may have occurred during rheumatism or chorea or following an attack of tonsilitis or from unknown cause. To the rough and at times calcified valves is assigned the greater susceptibility of these individuals to this infection. It is reasonable to suppose that this may aid in the localization of the microorganisms, but it does not, it seems to me, offer any explanation why these cases should progress steadily to a fatal end. The increased susceptibility of rabbits to subsequent inoculations with the cocci from chronic infectious endocarditis suggests that in the case of these patients there also exists an increased susceptibility. The primary attack of endocarditis may alter the mechanism of immunity in man just as the first injection appears to do in rabbits, in such a way that the cocci, tho not virulent in the usual sense, eventually gain the upper end.

CONCLUSIONS.

The following are points that seem to merit specific emphasis:

In chronic pneumococcus endocarditis the serum has no pneumococcidal power by itself.

The phagocytic power of the patient's blood is usually a better index of the actual conditions than the opsonic power of the serum determined with normal leukocytes and even with patient's leukocytes. The destructive power of the patient's blood as compared with that of normal blood corresponds well with the clinical conditions. Hence less reliance should be placed on the results of the opsonin determinations in which no account is taken of the fate of the ingested cocci.

The phagocytic power, as well as the destructive power, of the leukocytes in chronic infectious endocarditis shows greater variation in their own serum than in normal serum. Variations in the opsonic

power of the serum sometimes occur independently of variations in the phagocytic and destructive power of the leukocytes, but the variations on the part of the leukocytes is always dependent on changes in the serum.

While the chief action of serum in phagocytic mixtures is on the bacteria, there is no question, from the evidence at hand, but that the serum also exerts a definite and sometimes a striking influence on the leukocytes. Intraleukocytic destruction is dependent on a substance or property always present in normal serum and in normal leukocytes after treatment with normal serum, but frequently absent in the serum and leukocytes in chronic cases of endocarditis. At times the patient's serum is not only lacking in this substance, but contains another substance which seems to neutralize that in normal serum. This substance is not taken up by the bacteria but the opsonin or some other substance contained in washed sensitized pneumococci is necessary to activate it, because the cocci which have undergone spontaneous phagocytosis fail to show digestion.

In phagocytic mixtures disintegration of leukocytes is directly proportional to the amount of intraleukocytic destruction of pneumococci. Phagocytosis alone, even tho marked, does not seem to injure the leukocytes in any way.

The bacteria isolated from the cases shown appear to be modified pneumococci and prolonged cultivation in normal serum not only renders them largely resistant to phagocytosis, but makes them also resistant to intraleukocytic digestion. Both these properties are promptly lost in artificial media.

AN OUTFIT FOR SENDING BILE, SPECIMENS OF BLOOD, FECES, AND URINE, AND SOME RESULTS OF THE EXAMINATION OF SUCH MATERIAL.*

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In order to enable the physician to make an early diagnosis in typhoid fever, and also to detect the presence of typhoid bacilli in the feces and urine of convalescents and carriers, we have been using two outfits. One of these has been submitted to the postal authorities and permission has been granted to use it.

The specimen for examination is placed in 5 c.c. of Conradi's bile medium in a test tube, stoppered with a rubber stopper, and covered with a rubber cap. This test tube is then placed in a cylindrical tin box made of I. C. bright tin plate with soldered joints. This box is closed by a metal screw cover and a rubber or felt washer. The test tube in the box is completely and evenly surrounded on all sides by closely packed absorbent cotton. The box with its contents is now placed upside down in a larger box prepared in exactly the same manner except that it is lined on the inside with compressed paper not less than $\frac{3}{16}$ of an inch in thickness to hold the inner box snugly (Fig. 1).

For work within the city limits we use a simpler outfit which cannot be sent through the mail. These outfits are distributed bimonthly to our culture stations; the physician inoculates the tube of bile medium with blood, feces, urine, or other material and sends it by a messenger to the laboratory. The tube containing bile medium has a rubber stopper and is wrapped in cotton and placed in a cylindrical pasteboard box with a screw top.

Both of these outfits also contain a swab for inoculating the bile medium with feces,[1] and an aluminum box in which drops of blood can be collected for tests, according to the Wesbrook method.

* Received for publication February 10, 1910.

[1] *Directions for cultures from the feces and urine of cases of typhoid fever, paratyphoid infections, epidemic dysentery, and convalescent or carrier cases from such diseases.*—Fifteen drops of urine should be put in the bile tube in order to detect the presence of the bacteria in this fluid. In order to take a culture from the feces, simply obtain about 1 gram or 15 grains of the feces on the cotton swab which will be found in the outfit. Rub this up in the bile and then burn the swab. Place the bile tube in the outfit in the same way it was found, and send to the department. Wrap the bile tube carefully in cotton before replacing it in the case. Please fill out the blanks to be found in the outfit.

The drops of blood are collected on the inner side of the top of the box and must be thoroughly dried before the lid is put on. The bottom of the box contains four cover-glasses for blood smears.[1]

By the use of these outfits the physician loses none of the advantages of the ordinary agglutination and blood-smear tests; on the other hand the bacillus of typhoid fever may be detected in the blood or feces before the agglutination test is positive. The typhoid bacillus is present in the blood in at least 90 per cent of the cases during the first week of the disease; consequently before agglutination

FIG. 1

appears. The outfits can also be used to determine when convalescents are free from typhoid bacilli, and there is just as much reason for examining the feces and urine of the convalescent typhoid patient as for making throat cultures from convalescent diphtheria patients.

[1] *Directions for obtaining a blood culture in typhoid fever and paratyphoid infection.*—In order to obtain a blood culture in cases of typhoid fever or paratyphoid infection, the finger-tip or lobe of the ear should be carefully washed and then disinfected with 1–1000 bichloride of mercury or 5 per cent carbolic acid. A large surgical needle, or better a regular blood stilet, should be used, after sterilizing, to puncture the finger or ear lobe. Ten drops of blood should then be allowed to flow into the tube of bile, which will be found in the case. The tube should then be stoppered as before, placed in the case with the rubber stopper up, and the outfit should be sent to the department. The typhoid bacillus is frequently found in the first week of the disease, when the Widal reaction is still negative. An early diagnosis may be thus secured.

They can be used also in searching for typhoid carriers in institutions and houses in which other causes of typhoid fever have been eliminated, and we have already been able to solve several such problems by this means.

We believe that in the future managers of large hotels, superintendents of various institutions in which many persons are gathered, dairymen, and even careful housewives will wish to know whether cooks or persons handling raw food harbor typhoid bacilli, and it is not inconceivable that certificates, showing that the feces and urine are free from typhoid bacilli, will be demanded of such persons.

By means of the outfits described we have studied 279 bile cultures which were sent to the laboratory by physicians. Four of these were from one case of cholecystitis, 62 from urine, 39 from stools, and 174 from the blood.

As stated, the materials for examination are placed by the physician in tubes containing 5 c.c. of sterile bile medium. Fifteen drops of urine and about one gram of feces are recommended as suitable quantities for inoculation. The tubes are incubated for 24 hours, when plates are made with Wurtz's litmus lactose agar. In a sterile Petri dish drops of broth are inoculated with individual colonies. Those consisting of gram-negative bacilli are mixt in hanging drops with antityphoid serum which agglutinates in a dilution of 1:100,000. A dilution of 1:10,000 is used for testing agglutination of suspected bacilli. Colonies agglutinated within two hours are considered to be typhoid colonies and inoculations are made in litmus milk, gelatin, potato, and glucose, lactose, and saccharose broth in fermentation tubes. All colonies giving positive results when tested in this manner so far have been found to give the cultural reactions characteristic of typhoid bacilli in these media.

Urine.—The typhoid bacillus has been isolated from the urine by a number of observers. It is seldom found before the end of the second week of the disease and may very rarely persist in the urine for years, Young[1] having demonstrated the organism in a case of post-typhoid cystitis seven years after the patient had recovered from typhoid fever, and Hunner[2] and Simon[3] each twenty years after the attack. Petruschky[4] found that the excretion of typhoid bacilli through the urine of typhoid patients is

[1] *Johns Hopkins Hosp. Rep.*, 1900, 8, p. 401.
[2] *Bull. Johns Hopkins Hosp.*, 1899, 10, p. 163.
[3] *Klin. Jahrb.*, 1907, 17, p. 362.
[4] *Centralbl. f. Bakt.*, Abt. 1, 1898, 23, p. 577.

relatively rare, and that as they are never found at the beginning of the disease their demonstration in the urine cannot be used as a diagnostic method. He also found that the excretion of typhoid bacilli in a few cases reached millions of organisms in 1 c.c. of urine, and persisted for weeks, hence this is of the greatest importance in the prophylaxis of typhoid fever. In 50 urines examined the typhoid bacilli were found three times. In one of the cases the bacilli were first observed at the beginning of the third week and persisted for two months. In the other two cases they appeared in the urine in from 6 to 10 days after the fever had disappeared and were present for 4 to 8 weeks respectively. In one case they were followed by a hemorrhagic nephritis. Richardson[1] in 38 cases found typhoid bacilli in the urine in nine, or 24 per cent, but only in the third week of the disease. Horton-Smith[2] reports a case of pyuria, beginning on the thirtieth day and lasting throughout a relapse, in the urine of which were many typhoid bacilli. Of 12 cases examined he found the organism present in the urine in only two. He found the length of time the bacilli persisted in the urine in 10 cases to be 8, 21, 25, 30, 70, 13, 18, 19, 34, and 40 days. The figures up to 13 are absolute, showing when the bacilli disappeared, but from 13 on, all that is known is that cultures were obtained up to the time indicated. The average duration, therefore, was 30 days after the temperature was normal.

Herbert[3] examined 228 urines of 98 convalescents and found 18, or about 18 per cent, with typhoid bacteria, but Thomas[4] in 196 convalescents found only seven whose urine contained typhoid bacilli.

Petruschky, Richardson, and Horton-Smith all failed to find the typhoid bacillus in the urine until after the fifteenth day, and Hiss[5] examined 75 cases during the first two weeks of the disease without finding typhoid bacilli in the urine of any. It can be safely stated, therefore, that typhoid bacilli appear in the urine only after the beginning of the third week, and even then they are found in only about 7 per cent of the cases, as seen in Table 1.

Even in convalescence they are not frequently found, the average of the different authors (Table 2) giving only 6 per cent of positive results. Herbert, by very exact observations in hospital cases, found that the typhoid bacilli disappear from the urine in from 8 to 27 days after the temperature has reached the normal, the average being about 15 days. The paratyphoid bacillus occurs in the urine in apparently 4 per cent of cases, and in 3 per cent of convalescents.

We have studied cultures from 62 specimens of urine inoculated in bile medium and have found the typhoid bacillus only once and the paratyphoid bacillus once. Both patients were convalescent. A number of the cultures studied were from patients who never had typhoid fever.

Stools.—It was formerly believed that the stools of typhoid patients were always swarming with the causative bacillus. This belief was in part due to the fact that the earlier bacteriologists claimed to have found the organisms without difficulty in nearly all stools from typhoid patients. Since the introduction of the agglutination test and the various differential culture media by Hiss, Drigalski-Conradi,[6] Hesse,[7] and others,

[1] *Jour. Exper. Med.*, 1898, 3, p. 349.
[2] *Lancet*, 1899, 1, p. 1346.
[3] *Münch. med. Wchnschr.*, 1904, 51, p. 472.
Klin. Jahrb., 1907, 17, p. 207.
[5] *Med. News*, 1901, 78, p. 726.
[6] *Ztschr. f. Hyg.*, 1902, 39, p. 283.
[7] *Ibid.*, 1908, 58, p. 641.

the isolation of the typhoid bacillus from the stools has become much simplified and its recognition easy. Table 2 shows, however, that in 5,844 specimens of typhoid stools examined by various authors during the disease the bacillus was present 1,185 times, or in 20 per cent of the cases. Simon[1] examined 870 stools and Müller and Gräf 255 stools and noted the day of the disease of each examination, and Table 3 shows the appearance of the bacilli according to the weeks of the disease.

TABLE 1.

THE OCCURRENCE OF TYPHOID AND PARATYPHOID BACILLI IN URINE.

A. TYPHOID BACILLI IN URINE DURING THE ATTACK.

Author	No. Cases Examined	Positive	Percentage
Thomas*	267	13	4
Hiss	75	0	0
Petruschky	50	1	2
Richardson	38	9	24
Horton-Smith	12	4	33
Blumer†	10	1	10
Müller and Gräf‡	170	17	11
Total	622	45	7

B. TYPHOID BACILLI IN URINE DURING CONVALESCENCE.

Author	No. Cases Examined	Positive	Percentage
Thomas*	306	11	3
Herbert	228	18	7
Hiss	104	7	6
Petruschky	50	2	4
Blumer†	10	1	10
Müller and Gräf‡	46	6	13
Total	744	45	6

C. PARATYPHOID BACILLI IN URINE DURING THE ATTACK.

Author	No. Cases Examined	Positive	Percentage
Thomas*	216	4	1.5
Müller and Gräf‡	43	7	16
Total	259	11	4

D. PARATYPHOID BACILLI IN URINE DURING CONVALESCENCE.

Author	No. Cases Examined	Positive	Percentage
Thomas*	72	2	2
Müller and Gräf‡	20	1	5
Total	92	3	3

* *Klin. Jahrb.*, 1907, 17, p. 207.

† *Johns Hopkins Hosp. Rep.*, 1895, 5, p. 327.

‡ *Centralbl. f. Bakt.*, Abt. 1, Orig., 1907, 43, p. 856.

It is important from the standpoint of preventive medicine to determine how frequently the stools contain typhoid bacilli after convalescence has set in, and Table 3 also shows the percentage of positive findings after the temperature has become normal. In 703 cases examined the typhoid bacillus was found 19 times, or in 2 per cent of instances. This table also shows that the paratyphoid bacillus was found 33 times in 952 cases during the disease, or in 5 per cent.

Pratt, Peabody, and Long have collected 842 cases from various authors in which the typhoid bacillus was found in the stools 513 times. Some of these cases are included in our table.

[1] *Klin. Jahrb.*, 1907, 17, p. 232.

Table 3 indicates that the bacilli are present in the stools oftenest in the second week of the disease, quite frequently in the first week, and begin to disappear after the third week.

TABLE 2.

The Occurrence of Typhoid and Paratyphoid Bacilli in the Stools.

Author	No. of Cases	Positive	Percentage
A. Typhoid bacilli in stools during the disease.			
Thomas	895	51	5
Krause*	360	230	64
Drigalski†	384	75	19
Simon‡	3,150	508	16
Pratt, Peabody, and Long¶	206	43	21
Klinger§	173	68	39
Lentz and Tietz**	180	20	11
Hiss	118	45	38
Krause and Stertz††	104	53	51
Hoffman and Ficker‡‡	19	15	78
Müller and Gräf	255	77	30
Total	5,844	1,185	20
B. Typhoid bacilli in stools during convalescence.			
Pratt	21	0	0
Thomas	466	15	3
Herbert	216	4	1.5
Total	703	19	2
C. Paratyphoid bacilli in stools during the disease.			
Thomas	895	12	1
Müller and Gräf	57	41	70
Total	954	53	5
D. Paratyphoid bacilli in stools during convalescence.			
Müller and Gräf	31	12	3.5

* *Centralbl. f. Bakt.*, Abt. 1, Orig., 1904, 55, p. 723.
† *Klin. Jahrb.*, 1907, 17, p. 232 (cited by Thomas).
‡ *Ibid.*, 1907, 17, p. 229.
¶ *Jour. Amer. Med. Assoc.*, 1907, 49, p. 846.
§ *Arb. a. d. kais. Gesundh.*, 1906, 24, p. 35.
** *Munch. med. Wchnschr.*, 1903, 50, p. 439.
†† *Ztschr. f. Hyg.*, 1903, 44, p. 469.
‡‡ *Hyg. Runds.*, 1904, 14, p. 1.

We have studied 39 cultures from the stools. The typhoid bacillus was isolated from 11, or in 28 per cent of the cases, and the paratyphoid bacillus in 9, or 23 per cent of the cases. From one case both the typhoid and paratyphoid bacillus were isolated. Five of the positive results concern carriers, one having had typhoid fever 15 years before, a second 8 years before, a third 7 months before, and in two there was no history of typhoid. Outbreaks of typhoid were

traced to both of these cases, however, and as pointed out by Busse[1] it is probable that some bacillus carrriers do not develop typical typhoid fever even when the bacilli get into the blood. He obtained the bacilli from the blood of four persons with a severe infectious disease, but without any sign of typhoid fever during life or at autopsy.

TABLE 3.

THE OCCURRENCE OF TYPHOID BACILLI IN THE STOOLS ACCORDING TO WEEKS.

Week	Simon		Müller and Gräf		Total		Percentage
	No. of Cases	No. of Positives	No. of Cases	No. of Positives	No. of Cases	Positive Results	
1	84	8	42	13	126	21	16
2	176	35	78	29	254	64	25
3	165	30	42	13	207	53	20
4	120	15	16	4	136	19	14
5	81	7	10	3	91	10	10
6	58	5	8	1	66	6	9
7	37	2	11	1	48	3	6
8	33	1	9	0	42	1	2
9	31	1	2	1	33	2	6
10	20	1	4	0	24	1	4
11	14	1	1	1	15	2	13
12	8	0	2	0	10	0	0
13	10	1	1	0	11	1	10
14 to 21	33	1	1	0	34	1	3
	870	108	227	66	1,097	184	16

Blood.—In 1884 Gaffky[2] isolated the typhoid bacillus from the spleen in a case of typhoid fever and expressed the possibility of its being present in the blood. Castellani[3] was the first to isolate the typhoid bacillus from the blood during life. Since then the organism has been secured from the blood by many investigators, and the observations were collected by Coleman and Buxton[4] in 1907. We have added a number of cases from the more recent literature, bringing the data up to the present, the results being embodied in Table 4. Much of the earlier work concerned the question whether typhoid fever is a true septicemia and large quantities of blood were used in making the cultures. In 1901, however, Conradi[5] showed that bile possesses the property of keeping blood fluid, and in 1906 he proposed his method for hastening the growth of the bacilli in small quantities of drawn blood so as to make the culture available for diagnostic purposes.

Table 4 shows that in 2,359 blood cultures by various workers the typhoid bacillus was found in 1,625 instances, or in 68 per cent. Table 5 gives the percentage of positive blood cultures according to weeks. It shows that in the first week the typhoid bacillus was isolated from the blood in 78 per cent, in the second week in 69 per cent, in the third week in 57 per cent, in the fourth week in 32 per cent, and after the fourth week

[1] *Münch. med. Wchnschr.*, 1908, 55, p. 1113.

[2] *Mit. a. d. kais. Gesundh.*, 1884, 2, p. 372.

[3] *Centralbl. f. Bakt.*, Abt. 1, Orig., 1902, 31, p. 477.

[4] *Amer. Jour. Med. Sci.*, 1907, 133, p. 896.

[5] *Münch. med. Wchnschr.*, 1906, 53, p. 2387.

in 25 per cent of the cases. Müller and Gräf found the bacillus in the blood three times on the second, two times on the third, four times on the fourth, and once on the fifth day of the disease.[1]

TABLE 4.

SHOWING THE OCCURRENCE IN THE BLOOD OF

A. TYPHOID BACILLI.

Author	No. of Cases	Positive Results	Percentage
Coleman and Buxton......	1,602	1,197	75
Conradi..................	60	21	35
Castellani................	12	12	100
Peabody*................	33	24	72
Veil†....................	210	206	98
Müller and Gräf..........	360	110	30
Peabody‡................	82	55	62
Total................	2,359	1,625	68

B. PARATYPHOID BACILLI.

Author	No. of Cases	Positive Results	Percentage
Schottmüller§............	1	1	100
Conradi..................	60	3	5
Veil.....................	210	13	6
Müller and Gräf..........	360	10	2
Total................	631	27	4

* *Arch. Int. Med.*, 1908, 1, p. 149.

† *Deut. med. Wchnschr.*, 1907, 23, p. 1450.

‡ *Jour. Amer. Med. Assoc.*, 1908, 51, p. 978.

¶ Statistics collected by Coleman and Buxton.

§ *Münch. med. Wchnschr.*, 1904, 51, p. 294.

TABLE 5.

THE OCCURRENCE OF TYPHOID BACILLI IN BLOOD CULTURES ACCORDING TO WEEKS.

AUTHORS	FIRST WEEK			SECOND WEEK			THIRD WEEK			FOURTH WEEK			AFTER FOURTH WEEK		
	No. Cases Examined	Positive	Percent-age	No. Cases Examined	Positive	Percent-age	No. Cases Examined	Positive	Percent-age	No. Cases Examined	Positive	Percent-age	No. Cases Examined	Positive	Percent-age
Coleman and Buxton......	224	200	89	484	353	73	268	178	73	103	39	39	58	15	26
Peabody.......	5	5	100	19	15	78	9	4	44						
Veil...........	32	24	78	105	66	63	44	19	43	20	4	20			
Peabody.......	17	17	100	37	26	70	28	12	42						
Müller and Gräf........	57	17	29	96	44	45	36	10	27	19	3	15	16	4	25
Total	335	263	78	743	504	69	385	223	57	142	46	32	74	19	25

In our examinations of 174 cultures made from the blood the typhoid bacillus was isolated from 42 specimens, the paratyphoid bacillus from two, the pyocyaneus bacillus from two, and the colon

[1] Müller and Gräf's cases, like our own, were made from specimens sent by physicians to a municipal laboratory. The others were mostly made from hospital cases.

bacillus from one. From 22 cultures gram-staining micrococci were obtained; several of these proved to be the Staph. albus, probably derived from the skin of the patient in drawing the blood. One hundred and six of the specimens were sterile culturally and gave no agglutination, so we may consider our 47 positive cultures as coming from 68 cases, giving a positive percentage of 69.1. Cultures were made from the first up to the ninetieth day of the disease. The earliest positive cultures were obtained from two cases of two days' duration. Four positive cultures were obtained on the third day of the disease, six on the fourth, and three on the fifth day. Twenty-four or 51 per cent of the positive cultures were obtained in the first week of the disease, nineteen or 40 per cent in the second week, and three or 6 per cent in the third week, and in three the duration was not secured.

In 62 of the cultures the bile was incubated over night and the method recommended by Peabody of inoculating the water of condensation in a tube of blood serum was followed, and controlled by making plates with Conradi-Drigalski medium. The typhoid bacillus was recovered from the plates of 18 of these cultures, but in only nine instances did we secure a motile organism from the water of condensation in the blood-serum tube, and in one of these the organism was a gram-positive bacillus. The remaining cultures were made by merely incubating the bile tube over night, inoculating Wurtz agar, and making plates and incubating for 24 hours. At the end of this time the typhoid-like blue colonies were suspended in a few drops of sterile broth in a previously marked sterile Petri dish, the organisms from each suspension stained, and if gram-negative, hanging drops were made with antityphoid serum (agglutination strength of 1:100,000) at a dilution of 1:10,000, as well as a control drop without serum. If at the end of two hours agglutination and cessation of motility had occurred, the case was reported as typhoid. All positive colonies were inoculated into litmus milk, gelatin, potato, and glucose, lactose, and saccharose broth in fermentation tubes and, as stated, in every instance the bacilli gave all the cultural characteristics of the typhoid bacillus.

By this method we are able to make reports to the physician within 48 hours after receiving the specimen, and while the method

is not so rapid as the agglutination test, it must be emphasized that many of the positive cultures were obtained on the second and the third day of the disease.

When we meet with bacilli that do not agglutinate, we often feel that we must wait another 24 hours until it can be shown that they are not gas formers. In order to save this time we now inoculate glucose and lactose fermentation tubes with the bile after it has been incubated for 24 hours, and can thus rule out the paratyphoid and other gas-producing organisms at the same time that the agglutination of colonies is studied, namely, in 48 hours.

In seven cases the typhoid bacillus failed to agglutinate with immune serum or known typhoid blood. Our outfits always contain a specimen of the patient's own blood, however, and we use this also for testing the bacillus isolated from the bile culture. In all of these cases the bacillus was agglutinated with the patient's own blood. This test should always be carried out in order to aid in the identification of the organism. After several transfers to laboratory media four of the strains were agglutinated with known typhoid blood and immune serum (see Table 6).

The cultures from the pus in the case of cholecystitis, which is included in our series and which developed about three months after the patient had typhoid fever, gave typhoid bacilli in pure culture on each of four occasions.

It is interesting to note that this method will distinguish rare infections caused by B. pyocyaneus and B. coli from typhoid and paratyphoid fever, as strains of B. pyocyaneus and one of colon bacillus agglutinated with the patient's own blood. The paratyphoid bacilli isolated also agglutinated with the patient's own blood at a dilution of 1:50, or 1:200 of dried blood.

We have been able to find only one other instance in which B. coli was isolated from the blood during life, that of Czerny and Moser,[1] in which it was obtained from the blood of an infant.

The occurrence of primary pyocyaneus septicemia has been questioned by some who claim that this bacillus only appears in the blood following some more severe infection in which the general systemic resistance is lowered. Our cases in which the bacillus was obtained

[1] *Jahrb. f. Kinderh.*, 1894, 38, p. 430.

from the blood on the fourth day of the disease would seem to contradict this idea; in the second case agglutination was obtained on the tenth day of the disease. We are, therefore, led to believe that pyocyaneus bacillemia not only may be a primary disease but that further studies by means of blood cultures will show it to be more

TABLE 6.

AGGLUTINATION OF ATYPICAL TYPHOID BACILLI AND OTHER ORGANISMS.

CULTURE NO.	AGGLUTINATION	DURATION OF DISEASE	ORGANISM ISOLATED FROM CULTURE	DAY OF AGGLUTINATION			
				P.B.	T.B.	A.S.	PT.B.
126	0	14 days	B. coli	1	0	0	0
133	0	4 "	" typhosus	1	1	1	0
133	0	4 "	" paratyphosus	1	0	0	1
144	0	4 "	" pyocyaneus	1	0	0	0
167	0	21 "	" typhosus	1	0	0	0
174	0	10 "	" "	1	0	0	0
188	0	8 "	" paratyphosus	1	0	0	1
203	0	14 "	" typhosus	1	2	2	0
213	0	3 "	" "	1	0	2	0
231	0	10 "	" "	1	0	4	0
256	0	3 "	" "	1	0	0	0
259	0	14 "	" pyocyaneus	1	0	0	0
272	0	8 "	" typhosus	1	4	4	0

P.B. = patient's blood; T.B. = known typhoid blood at a dilution of 1:50; A.S. = antityphoid serum at a dilution of 1:10,000; PT.B. = known paratyphoid blood at a dilution of 1:50; figures denote the days of agglutination after organism was isolated.

common than generally believed at present. Our view is strengthened by the fact that in the literature are found several cases in which the clinical symptoms closely resembled those of typhoid fever, but which at autopsy showed no evidence of the latter disease and from which B. pyocyaneus was isolated. Cases of this kind are recorded by Channin, Ehlers, Oettinger. As shown by Waite who gives a thorough review of the literature on pyocyaneus infection,[1] B. pyocyaneus has been found in all organs after death, but only in seven authentic cases do we find that it was isolated from the blood during life (Blume, Boinet, Brill and Libman, Eastman and Keene, Finkelstein, Heubener, Rolly).

The agglutinin concentration in the blood in typhoid and paratyphoid fever.—Müller and Gräf have tested carefully the maximum dilution at which the blood of cases of typhoid fever would cause agglutination. They studied 203 cases, and found that the maximum dilution in 9 cases was 1:30, in 37 cases 1:50, in 37 cases 1:100, in 90 cases 1:200, in 8 cases 1:500, in 10 cases, 1:1000, in 10 cases 1:2000, in 1 case 1:2500, and in 1 case 1:5000. They also studied 28 cases of paratyphoid infection which gave no agglutination of typhoid bacilli. In these cases the maximum dilution

[1] *Jour. Infect. Dis.*, 1908, 5, p. 542.

was 1:100 in 3 cases, 1:200 in 9 cases, 1:500 in 8 cases, 1:1,000 in 2 cases, 1:2,000 in 4 cases, and 1:5,000 in 2 cases. Furthermore, they studied 14 cases of paratyphoid infection from which the organism was isolated; in 11 of these cases paratyphoid bacillus was agglutinated in much higher dilution than the typhoid bacillus; in the remaining three the reverse conditions obtained. All of the cases, however, gave a positive reaction with the typhoid bacillus in low dilutions.

The investigators also find that the blood from a typhoid fever patient will show great differences in the agglutinin strength with respect to different strains of typhoid bacilli. They examined 63 cases in which the maximum dilution was higher for one strain than for others. Some strains are but slightly agglutinable. In our work we made comparative tests of a large number of strains and selected for routine diagnosis one which agglutinated with maximum dilution in immune serum and typhoid blood.

CONCLUSIONS.

Physicians should use the bile-medium method in order to secure an early diagnosis in intestinal infections, as well as to determine when the various excretions are free from typhoid and paratyphoid bacilli.

Persons in large institutions who handle raw foods should have their feces and urine examined for typhoid bacilli, especially if there is a history of previous intestinal infection.

The bile method as here recommended will enable physicians to secure an early diagnosis of many of the intestinal infections by making cultures from the feces and blood. Rarer septicemias caused by B. pyocyaneus and other bacteria may also be detected.

BACILLUS ABORTUS* OF BANG, THE CAUSE OF CONTAGIOUS ABORTION IN CATTLE.†

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The existence of a contagious disease of cows, the most characteristic manifestation of which is the premature delivery of the fetus, has been recognized by veterinarians and by practical husbandmen for a long time. The transmissibility of this disease was proven experimentally by Brauer[1] and has been abundantly confirmed. The detection of the specific cause and its artificial cultivation has seemed to offer special difficulties, as may be judged from the painstaking but inconclusive work of Nocard[2] in France and of Moore[3] in this country. In 1897 Bernhard Bang[4] found in the uterine exudate of a cow, slaughtered during an attack of this disease before the abortion had taken place, a small short bacillus with very interesting biological properties, which he was able to grow in pure culture, and by the inoculation of which he was able to produce the disease in healthy animals. The usual methods of artificial culture failed to produce cultures of this organism. Bang and Stribolt obtained growth by inoculating the uncontaminated uterine exudate obtained after slaughter into a serum gelatin agar. The medium employed was the usual meat water peptone broth to which had been added 0.75 per cent agar and 5 per cent gelatin. This was melted and cooled to about 45° C. and then mixt with about half its volume of sterile blood serum. While still fluid, the medium was inoculated and dilutions made in a series of test tubes in the ordinary way. After mixing, the medium was solidified by immersing the tube in cold water and was

* The germ has been named Abortusbacillus by Bang and Bacterium abortivum by Chester (*Manual of Determinative Bacteriology*, New York, 1901, p. 121). The name Bacillus abortus has been adapted by transposition of that given by Bang. The specific name abortus, being in the genitive case, may be properly employed with either of the generic terms, Bacillus or Bacterium.

† Received for publication February 16, 1910.

[1] Cited by Ostertag, Kolle und Wassermann, *Handbuch*, 3, p. 825.

[2] Cited by Bang, *Ztschr. f. Thiermedicin*, 1897, 1, p. 241.

[3] *Amer. Vet. Med. Assoc. Rep. of E. V. Wilcox*, Exper. Sta. Rec., 1904–5, 16, p. 122.

[4] *Op. cit.*

then incubated at 37° C. The colonies of the bacillus developed in such tubes only in a particular zone beginning about 5 mm. beneath the surface of the medium and extending down for 1 to 1.5 cm. Below this region the medium remained clear. Bang concluded therefore that the bacillus is neither an aerobe nor an anaerobe in the usual sense, but exhibits a very peculiar behavior in respect to oxygen, requiring a partial pressure of oxygen less than that present in the atmosphere, and is unable to grow in the presence of the ordinary air, or in the absence of oxygen. This interesting feature was very fully examined by Bang and Stribolt and they found that there are two optima in the partial pressure of oxygen, one somewhat less than the partial pressure of atmospheric oxygen, and the other the partial pressure of nearly pure oxygen (about 90 per cent). A tube culture on serum gelatin agar incubated in pure oxygen or in ordinary air under a pressure of five atmospheres gave rise to two zones of colonies, one near the surface of the medium and the other near the bottom of the tube. Between them the medium remained clear. Good cultures of the bacillus were also obtained in serum glycerin broth in an atmosphere of nearly pure oxygen.

With these cultures Bang has been able to produce the disease in cows by application to the vaginal mucous membrane, by intravenous injection, and by feeding. He has also infected sheep, goats, rabbits, and one mare, producing abortion and recovering the bacillus from the uterine exudate in each case.

Nowak[1] has confirmed the work of Bang, and placed practically beyond question the etiological relation of the bacillus to the disease. The particular advance which he has made, however, is the discovery of a practicable plate method for the isolation of the abortion bacillus from contaminated material. For this purpose ordinary agar is melted and cooled to 50° C., then mixt with about one-fourth its volume of naturally sterile blood serum, and poured into sterile Petri dishes where it is allowed to solidify. The piece of the placenta or other material from the abortion is now streaked over several of these plates in succession, according to the usual bacteriological technic for obtaining streak dilution cultures. The plates are then placed at 37° C. for 24 hours to allow contaminating aerobic bacteria to

[1] *Ann. de l'Inst. Pasteur*, 1908, 22, p. 541.

develop. Parts of the plate surface free from colonies are now marked with a wax pencil, as it is upon these areas that the growth of the abortion bacillus may subsequently appear if it is present. The plates are next put into a glass jar or desiccator together with a culture of the common hay bacillus, B. subtilis. About 1 sq. cm. of culture surface of the latter organism should be allowed for each 15 c.c. air capacity of the jar. The jar is now closed and placed at 37° C. for three days, at the end of which time the transparent colonies of the abortion bacillus will have developed, if it is present. The growth of the B. subtilis culture serves to absorb the oxygen in the jar to just the partial pressure required for the development of the abortion bacillus.

It may be accepted as certain that this germ is the microbic cause of contagious abortion in several species of domestic animals, but it is too early to conclude that it is the only germ causing a disease in which abortion is a prominent feature. In fact, Ostertag[1] has investigated an epidemic of contagious abortion in mares, in which he was unable to find this bacillus, but did find a streptococcus in every case.

In the United States, contagious abortion is a widespread disease of cattle, and recent investigations of the disease at the Arizona Agricultural Experiment Station have been reported by Wilson, and at the Connecticut Storrs Agricultural Experiment Station by Beach. So far the isolation of the bacillus of Bang appears not to have been reported in this country. In reporting our work at this time we purpose to add further confirmation to the work of Bang and to direct the attention of American bacteriologists to the very interesting biological properties of this bacillus discovered by him.

Case 1.—Cow. Abortion occurred at 5 A.M. July 10, 1908, at beginning of seventh month of pregnancy. The placenta was removed from the uterus by the herdsman and arrived at the laboratory at 10:30 A.M. Many of the cotyledons were yellowish-white in the centers, some were entirely broken down and purulent on the surface. A large area of the amnion was thickened and contained many small hemorrhages, and a few opaque yellowish areas. A small piece of purulent cotyledon was suspended in sterile broth and two small guinea-pigs (not pregnant) were inoculated, one subcutaneously, the other intraperitoneally. Neither guinea-pig was seriously affected.

Case 2.—Cow. Abortion, during the eighth month of pregnancy, occurred August 30, 1908. A portion of the placenta was kept in a refrigerator and examined on

[1] *Op. cit.*

September 3. The appearance of the placenta resembled that of Case 1. There was no offensive odor. Bits of the altered placental tissue were inoculated into blood broth fermentation tubes, and blood agar, blood gelatin, and plain agar stab cultures were made. All these cultures were incubated at 37° C. for 24 hours and by microscopic examination were found to contain a variety of bacteria. They were kept in the refrigerator until September 7 upon which day two pregnant female guinea-pigs were inoculated. The first received three drops of a blood broth fermentation tube culture subcutaneously. Nine days later, September 16, edema of the vulva was observed for the first time. On September 23 there was a purulent discharge at the vaginal outlet but the fetuses were still present in the abdomen. On September 25, two days later, the uterus was empty, there was no vaginal discharge apparent, and no sign of the products of conception in the cage. The animal was killed by chloroform and blood broth fermentation tubes were inoculated from the inside of the partly involuted uterus. These remained free from growth. The second guinea-pig received on September 7 three drops of the same blood broth culture into the vagina. No edema of the vulva or other pathological change was observed in this animal. On September 25, Caesarian section was performed under ether and two living and almost fully developed fetuses were removed. Fermentation tubes of blood broth were inoculated with exudate from the endometrium, with the amniotic fluid, and with bits of placental tissue. These gave no growth. Direct microscopic examination of the tissues was negative.

Case 3.—Heifer. Abortion occurred during the eighth month of pregnancy on March 17, 1909. A piece of the placenta was examined; it presented the appearance described in Case 1. Bits of the altered placental tissue were used to make streak inoculation on a series of six serum agar plates, according to the method of Nowak. Two stab cultures with Stribolt's serum gelatin agar were also made, and a pregnant female guinea-pig was inoculated subcutaneously with 0.5 c.c. of a suspension of a bit of placenta in broth. This guinea-pig aborted during the night of March 27 but none of the products of conception were found and no further work was done with the animal. Direct microscopic examination of cover-glass preparations of the cow's placenta showed few bacteria, but at least two different kinds were present, a ong bacillus and a very short bacillus.

The serum agar plates (Nowak) were incubated at 37° C. in the air for 24 hours and the colonies which developed were checked by a blue pencil mark on the outside of the bottom of the Petri dish. The dishes were then placed in a Novy anaerobe jar together with one Petri dish plate culture of B. subtilis,[1] the jar closed, and incubated four days at 37° C. On March 22 excellent colonies corresponding to the description and to the photographs of Nowak were found in rows on the portions of the plates formerly free from colonies. The colonies were made up of short non-motile bacilli without spores. Subcultures were made on inclined serum agar tubes, some of which were incubated in the air and others in the glass jar together with plates of B. subtilis. After three days the former set of tubes showed no growth, and on the latter excellent cultures had developed. Subcultures were again made from these, the remainder of the growth was suspended in sterile broth, and 0.5 c.c. of the suspension was injected subcutaneously into each of two pregnant female guinea-pigs (March 25). One of the guinea-pigs inoculated aborted during the night from March 28 to 29, only three and a half days after inoculation. The two fetuses were of nearly full size but both were dead. No placenta was found. At autopsy it was found that one of the

[1] Inoculated by overflowing the surface of the solidified agar with a broth suspension of the bacillus.

young guinea-pigs had not breathed, but in the other one the lungs were well expanded, the stomach filled with gas, and there was some gas in the upper part of the small intestine. The animal must therefore have lived a short time after birth. Serum agar plates were inoculated with the heart blood and the intestinal contents of each fetus but none of the specific bacteria were detected. The mother guinea-pig was chloroformed at 11 A.M. and autopsy performed at once. At the point of inoculation there was a hard, almost cartilaginous infiltration, the size of a quarter, and the skin over it was red in color. On section the subcutaneous tissue was yellowish-white in color and very firm. The bacillus of Bang was detected here in pure culture, microscopically, and by plate culture. Cultures inoculated with heart blood and with the serous fluid from inside the uterus remained sterile. In this animal, then, aborting 3½ days after subcutaneous inoculation with the bacillus, there was no proof that the infection had extended to the uterus.

The other pregnant female guinea-pig, inoculated subcutaneously on March 25 with a pure culture of the bacillus, aborted early on the morning of April 2, eight days after inoculation. At 8:00 A.M. two half-grown fetuses and two placentae were found in the cage with her, one of the latter having been partly eaten. B. abortus was found in each placenta by microscopic examination of cover-slip preparations, and also by plate cultures. It was not found upon the plates made from the fetal livers. The mother was chloroformed at 8:30 A.M. and autopsy performed at once. At the point of inoculation the skin was reddened and there was a very hard subcutaneous swelling. Upon section the corium was greatly thickened and infiltrated with pus, walled off below by a dense capsule of cartilaginous consistency. The bacillus was found here in pure culture by microscopic examination and by plate cultures. The bacillus was not found in the heart blood, but typical colonies developed on the plate cultures inoculated with uterine exudate.

From this cow's placenta (Case 3), then, was obtained a bacillus corresponding to the description of Bang and of Nowak, and by inoculations with a pure culture of this germ, premature labor was induced in guinea-pigs and the germ was recovered from the placenta and from the endometrium of one of these animals.

Case 4.—Heifer. Calf was still-born at term, April 9, 1909. The placenta and the body of the calf were examined, and a number of plate cultures made from them, but no B. abortus was isolated.

Case 5.—Cow. Living calf was born at term, April 12, 1909. Placenta was not obtained. A sample of vaginal discharge was brought to the laboratory April 14 and plate cultures made. B. abortus could not be isolated.

Case 6.—Heifer. Abortion occurred at fifth month on April 15, 1909. The animal had been carefully examined on the previous day before the class, and appeared perfectly healthy. Complete membranes and fetus were brought to the laboratory. B. abortus could not be isolated.

Case 7.—Cow. Abortion occurred April 21. Only a piece of amnion was saved for examination; no placental tissue. B. abortus could not be isolated.

Case 8.—Cow. Normal calf was born at term, May 3, 1909. Placenta was brought to the laboratory. It appeared normal. Plate culture failed to isolate B. abortus.

Case 9.—Cow. Normal calf was born at term, May 5, 1909. Placenta was not found. Vaginal discharge was examined by the plate method with negative results.

Case 10.—Cow. Premature delivery of a living calf occurred July 9, 1909, at the

beginning of the eighth month of pregnancy. Placenta was not obtained. Some of the discharge collected from the ground in an open field was brought to the laboratory. B. abortus could not be isolated by the plate method.

Case 11.—Cow. Abortion, at seventh month of pregnancy, occurred July 10, 1909. A piece of placenta was brought to the laboratory the next morning. Most of the cotyledons were grayish yellow and purulent. Five serum agar plates (Nowak method) were made, and on two of these pure colonies of B. abortus were obtained. The bacillus is identical in cultural characteristics with that obtained from Case 3. No animal inoculations have been made with it.

Case 12.—Pig. Sow aborted July 18, 1909. Two fetuses and a piece of the membrane, but no placental tissue, were brought to the laboratory. B. abortus could not be isolated.

Case 13.—Pig. Sow aborted during the night from July 28 to 29. One fetus and a piece of membrane with a bit of broken down placental tissue was brought to the laboratory July 29. B. abortus could not be isolated.

Case 14.—Cow. Living calf was born at term, September 24, 1909. Placenta was examined. B. abortus could not be isolated.

Case 15.—Cow. Living calf was born at term, September 25, 1909. Placenta was examined. B. abortus could not be isolated.

Case 16.—Cow. Living calf was born at term, September 29, 1909. Placenta was examined. B. abortus could not be isolated.

Case 17.—Cow. Living calf was born at term, October 3, 1909. Placenta was examined. B. abortus could not be isolated.

Case 18.—Cow. Living calf was born at term October 11, 1909. Placenta was examined. It was normal in appearance. B. abortus could not be isolated.

Case 19.—Cow. Living calf was born at term, November 8, 1909. Placenta was examined. B. abortus could not be isolated.

SUMMARY.

Of the 19 cases examined two were sows and 17 were cows. Of these latter, 10 were delivered at term. In the other 7 delivery was premature. Of these, one case, No. 6, was clinically not an abortion due to infection. This leaves six cases which were clinically cases of contagious abortion. Two of these, Cases 1 and 2, were examined before the plate method was employed, and cultures were negative. A guinea-pig inoculated from Case 2 aborted. Of the four remaining cases, No. 3 and No. 11 gave positive cultures of B. abortus from the placenta in each case. In Case 7 only a bit of membrane without placental tissue was available and in Case 10 only some of the discharge gathered from the ground. Both of these, examined by the plate method, gave negative results.

Subcutaneous inoculation into a pregnant female guinea-pig, of a broth culture of the mixt bacteria of a contaminated placenta (Case 2),

produced abortion in 26 days. Intravaginal application of the same material in another pregnant female guinea-pig was without result. Subcutaneous inoculation of a broth suspension of infected placenta (Case 3) into a pregnant female guinea-pig produced abortion in 10 days, and the subcutaneous inoculation of a pure culture of B. abortus isolated by the Nowak plate method from the same placenta caused a premature delivery after three and a half days in one guinea-pig and an abortion eight days after inoculation in a second guinea-pig. From this last animal the bacillus was recovered from the point of inoculation, from the interior of the uterus, and from each of the two placentae.

From this rather limited series of examinations and experiments we may conclude that the bacillus of Bang is the microbic cause of at least some of the contagious abortion of cattle in this country. We hope this report may prove of some value to workers in this particular field, and that it may also serve to draw the attention of others to the very interesting biological properties of this peculiar microorganism.

SOME OBSERVATIONS ON THE WASSERMANN REACTION.*

LAWRENCE T. CLARK.

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AN esteemed early teacher once said: "The bacteriologist of yesterday was one who isolated and cultivated bacteria and attempted to classify them; the bacteriologist of tomorrow must be one who cannot only grow these microorganisms, but measure the scope and products of their activities and offer means for correcting their evil effects." In the serum diagnosis of syphilis, wherein the phenomenon known as the fixation of complement is employed, the products of parasitic infection have been detected and measured before the bacteriologist has succeeded with the more fundamental procedure as the isolation and growth of the Treponema pallidum.

The value and importance of the complement fixation reaction to the clinician and, indirectly, to the public, and difficulties attending its extensive use have been repeatedly set forth. I believe these facts are sufficiently familiar to all to need no further comment at this time. Modifications of the original Wassermann method, substitutions for some of the various ingredients of this test, and reactions depending on different phenomena have been exploited by enthusiastic workers who have striven to perfect a test which would be within reach of the average clinician. In the main the use of these so-called modifications leaves the doubtful case still doubtful and gives definite results only where such a test is not needed. The competent practitioner requires aid from a reliable test in his doubtful cases only; those with a known history and those with clearly diagnostic symptoms need no verification. The more urgent need, then, is for a test upon which we can place absolute dependence in the doubtful case in which accurate diagnosis would mean much to the physician and more to the patient, to whom an error in diagnosis might bring lasting injury.

The questions then arise, how and by whom shall the serum test for syphilis be made? Can the practicing physician with a few cases

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each month carry out the original test or some reliable modification and do justice to his doubtful case?

Among the more commendable modifications employed by various workers, that of Noguchi[1] renders the test less complicated and, according to Fox,[2] more accurate than the original Wassermann. The necessary reagents, reduced to a more or less stable condition, are thus made available for making the test at the bedside if necessary.

During the past two years the author has tested 494 bloods by different methods and has arrived at a test, slightly modified from the original Wassermann, upon which he places considerable reliance. The first 392 samples were tested with precipitin reagents in various combinations for the precipitin reaction. It was hoped that a method simpler than that depending upon complement fixation could be worked out, which would give a reaction easy to read and reliable when carried out by the practicing physician. Results obtained with the 392 cases, the majority of which were diagnosed clinically, gave a percentage of less than 70 per cent of reliable findings. While the clinician having access to a large number of cases may be able to detect slight differences between questionable and normal bloods, the precipitin tests are too delicate for him who most needs such and has but few cases at his command.

The color test described by Schurmann[3] was also tried, but gave very unsatisfactory results. I was unable to confirm the findings of the writer.

Owing to lack of success with the methods tested and in order to control results obtained with precipitin reagents, the original Wassermann reaction was taken up and in a somewhat modified form has been used since. The five factors as employed are:

1. Patient's serum inactivated by heating at 56° C., ½ hr.
2. Antigen—alcoholic extrac of syphilitic liver.
3. Complement—fresh guinea-pig serum.
4. Hemolytic antigen—washed red corpuscles from sheep, diluted 1:100 in salt solution.
5. Hemolytic amboceptor—anti-sheep-corpuscle rabbit serum.

One standard drop of patient's serum, known normal serum, and known syphilitic serum were put into three separate tubes (5 cm.×0.8 cm.). To each of the three tubes one unit of antigen was then added and the tubes shaken; 0.02 c.c. of fresh normal

[1] *Jour. Exper. Med.*, 1909, 11, p. 392.

[2] *Jour. Cutan. Dis.*, 1909, 27, p. 338.

[3] *Deut. Med. Wchnschr.*, 1909, 35, p. 616.

guinea-pig serum was next added, the mixture shaken thoroughly and incubated 20 minutes. Immediately upon removing the tubes from the incubator 1 c.c. of sheep washed red corpuscles (1:100) and 1 unit (1/625 c.c.) inactive hemolytic serum were placed in the tubes. They were again shaken and allowed to stand 10 minutes at room temperature when readings were taken.

Several sets of tests, carried out according to this method with the complement from the same guinea-pig, gave entirely concordant results. Complete hemolysis took place in the normal controls within 10 minutes, while no hemolysis occurred in the syphilitic tubes. Believing at that time that the test as outlined was adapted to the purpose intended, I planned to run a number of samples independently to test the accuracy and uniformity of the method before comparing results with those obtained with precipitin reagents.

Samples of the serum bearing numerals or letters only were very kindly furnished me by Dr. H. R. Varney and Dr. Andrew D. Potter. Clinical data pertaining to cases were purposely withheld, consequently the laboratory tests were in no way influenced by them.

Difficulties were, however, encountered in changing the source of guinea-pig serum. The entire set of tubes frequently gave complete hemolysis, due to an excess of complement, or no hemolysis because of too little complement. In such instances the amount of complement was varied as the first results indicated, a retest made, and more reliable results were in that way obtained. Such a procedure is objectionable, as it exhausts the sample and takes more time than is necessary. My findings, as well as those of other workers, clearly indicating that fresh sera from different guinea-pigs possess decidedly variable amounts of complement, I at once undertook to correct this troublesome feature of the test and remove the necessity for making a retest.

A quantity of serum was obtained from a known active syphilitic whose serum had given good reactions and also from a normal individual whose serum had given no trace of a reaction by all methods employed. Sera were then obtained from a number of normal guinea-pigs of varying ages, and amounts varying from 0.02 to 0.04 c.c. taken to make the regular test as previously outlined. From these series of tests the amount of fresh guinea-pig serum necessary to cause complete hemolysis in 10 minutes with a normal serum was found to vary materially between 0.02 and 0.04 c.c.

From the above results this variation in that quality of guinea-pig serum subject to fixation accounts for the irregular results obtained when a constant amount of guinea-pig serum was used in earlier trials.

The limits, then, of the amount of complement to use in making a reliable test and the variation met with in the serum from different pigs call for a careful standardization of each lot of guinea-pig serum before using. While this procedure makes the test more complicated, the author has full confidence in the greater accuracy it gives.

To standardize the fresh guinea-pig serum, two series of three tubes each are taken; to one series one standard drop of inactive syphilitic serum is added per tube, and to the second series one standard drop of inactive normal serum is added per tube. One unit of antigen is then placed in each of the six tubes and shaken and 0.02, 0.03, and 0.04 c.c. of the guinea-pig serum is added to tubes 1, 2, and 3 of each series respectively, and all are then incubated 20 minutes. After incubation the hemolytic couple is added to all tubes and hemolysis watched for during the succeeding 10 minutes. The amount of guinea-pig serum necessary to give complete hemolysis in the normal tube with none in the corresponding syphilitic tube is then taken as the unit of complement in that particular guinea-pig serum and the regular test then carried out with the unknown samples. A convenient form of tube for measuring the guinea-pig serum was found in capillary pipettes, which were carefully graduated in hundredths by means of mercury.

One hundred and five sera were tested by the author's improved method just described. Of these samples 60 were from clinically diagnosed cases and 45 were doubtful; 57, or 95 per cent, of the known cases gave results corresponding to the clinical evidence; 42, or $93\frac{1}{3}$ per cent, of 45 cases of doubtful diagnosis gave results substantiated by subsequent clinical findings; 8 cases with a positive initial reaction gave succeeding negative reactions after vigorous specific treatment.

Concerning the keeping qualities of the several factors used in making the test, I find that the hemolytic serum kept constantly at 1.6° C. slowly loses its strength. A lot made nine months ago is at present in use. Its titre when first taken was 0.002, which changed

to 0.003, 0.004, and, finally at this time, is 0.009. I have successfully used corpuscles (1 per cent), which were kept 14 days at 1.6° C. suspended in isotonic salt solution. Lack of uniformity in results has caused me to discontinue using guinea-pig serum more than 24 hours old. No change has been detected in the strength of the specific antigen (alcoholic extract), which was extracted 10 months ago. The inactive patient's serum, if kept at refrigerator temperature and free from contamination, has given uniform results for lengths of time varying from 7 to 30 days.

CONCLUSIONS.

The complement content of fresh guinea-pig serum varies materially with different pigs.

More uniform and accurate results are obtained when the guinea-pig serum is standardized to known normal and syphilitic sera before doubtful samples are tested.

Hemolytic serum kept at uniform low temperature retains its activity for a relatively long time, altho it loses some of its original strength and needs restandardizing from time to time.

Suspensions of thoroughly washed red blood corpuscles (sheep) kept at 1.6° C. have been used up to 14 days after drawing with good results.

Samples of serum inactivated (56° C. one-half hour) and kept free from contamination remain unchanged for several days. This enables one to store samples and examine several at one operation.

Practical tests made with properly standardized reagents gave 95 per cent and $93\frac{1}{3}$ per cent accurate results in known and doubtful cases respectively.

Negative reactions were obtained after vigorous specific treatment in 8 cases which gave positive reactions before treatment. A future publication will deal with this phase more extensively.

It would seem to be indicated by results from the limited number of cases tested that the complement fixation reaction, when carefully carried out and thoroughly controlled, is a reliable means for diagnosis in doubtful cases.

THE INFLUENCE OF AGE AND TEMPERATURE UPON THE POTENCY OF ANTIDIPHTHERIC SERUM AND ANTITOXIC GLOBULIN SOLUTION.*†

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INTRODUCTION.

FOLLOWING the work of Ehrlich, the determination of the potency of diphtheria antitoxin has been placed upon an exact and scientific basis. All the diphtheria antitoxin manufactured in the United States is now standardized in accordance with the unit furnished by the Hygienic Laboratory, which was actually promulgated April 5, 1905. Following the adoption of the American unit, antidiphtheric serum was admitted to the Eighth Decennial Revision of the United States *Pharmacopoeia.*

Considerable work has been done upon the duration of passive immunity following the administration of diphtheria antitoxin. It seems to be now generally accepted that passive immunity, following the use of diphtheria antitoxin, cannot be relied upon longer than three or four weeks.

While there has been a large amount of work done upon the above two phases of the manufacture and use of diphtheria antitoxin, there has been comparatively little done upon another most important part of the manufacture of this therapeutic product, i.e., an exact determination of the influence of age and temperature upon the potency of diphtheria antitoxin.

It has been the custom of most American producers of diphtheria antitoxin to date their serum to be returned or exchanged for fresh antitoxin in from 9 to 12 months after being placed upon the market. Lately some manufacturers have extended this period to 18 months or even two years, without, however, having any very exact data as to the keeping qualities of diphtheria antitoxin under adverse conditions.

* Received for publication April 10, 1910.

† Read before the Laboratory Section of the American Public Health Association, Richmond, Va., October 20, 1909.

Consequently, there has been a demand on the one hand from the manufacturers of antitoxin for data on this subject for commercial reasons, and on the other hand from physicians from the standpoint of therapeutic efficiency. Some physicians even go so far as to doubt the propriety of using antitoxin, even in cases of emergency, when the return or exchange date on the package has been passed. I know of at least one instance in which a physician, in spite of the fact that he was not able to obtain fresh antitoxin, refused to use a package of serum on which the return date had expired.

Prior to 1906 there had been some work reported on the keeping qualities of diphtheria antitoxin, but most of it was open to the criticism either that the potency had not been determined in accordance with the Ehrlich method or that its strength had not been determined in the beginning or at the end of the experiment within sufficiently close limits to justify exact conclusions as to the influence of age and temperature. It would be manifestly unfair to draw conclusions as to the loss in strength of diphtheria antitoxin if a package of serum was placed upon the market containing an excess to allow for loss in potency, and when returned from the market and tested for its decrease in potency, not to take into account this excess. For example: If a package of serum labeled "to contain 1,000 units," but actually containing 1,200 units, or 20 per cent excess to allow for loss in strength, was placed upon the market and then returned at the end of a year and tested to ascertain whether it still contained 1,000 units, the package might be found to still contain 1,000 units of serum in spite of the fact that it had lost the 200 units excess placed in the package.

Early in the fall of 1906 the question of the deterioration of diphtheria antitoxin incident to age and temperature was taken up in the Hygienic Laboratory and all the licensed manufacturers in the United States were invited to contribute serum for this purpose, and several of them did so. In all, there were 18 different lots of serum furnished, 14 being untreated serum and 4 serum concentrated by the Gibson process. It is to be regretted that the number of both kinds of serum was not larger, but it is believed that the number was sufficient to provide data as to the influence of age and temperature upon the potency of antitoxin.

PREVIOUS WORK.

Alba[1] gives the results of retests of ten lots of diphtheria antitoxin and concludes that antitoxin retains its potency unchanged for a long time. His results are of little value to us, as we know that the method of testing the potency of serums, prior to the use of the present method of Ehrlich, was not accurate.

Marx[2] gives the results of the retesting of 1,104 samples of serum at the Institut in Frankfurt sent there between March, 1895, and June, 1903. Of this number, only 34, or 3.08 per cent, were withdrawn on account of a decrease in potency. The losses by months were as follows: in 3 to 4 months, 2 samples; 6 to 10 months, 9 samples; 10 to 20 months, 23 samples. He concludes that the loss in potency, when it does take place, is gradual and that the lack of confidence in old sera is not well founded.

An editorial in the *Journal of the Royal Army Medical Corps*[3] gives the results of the retesting of six lots of serum that had been for a year either in military hospitals at home or had made one or two voyages to India on transports during that time. Only four of the six lots had lost in potency, the loss varying from 5 to 18 per cent. The conclusion drawn was that antitoxin retains its activity little, if any, impaired at least 12 months.

Miller[4] tested the strength of a large number of packages of serum returned to the manufacturers. The time between tests varied from seven months to more than six years. Of 82 lots tested, 30 per cent showed a loss in potency varying from 0 to 50 per cent. It was found that the high potency serum underwent a greater decrease in potency than the low; but that every package, when retested, contained at least the number of units claimed on the label due to the excess allowed by the manufacturer for deterioration.

Layson[5] studied the decrease in potency of diphtheria antitoxin kept under known conditions from one to four years. His first tests were made on 14 lots of serum kept in bulk for one year at 4° C., then kept in a cool room (temperature not stated) for a year, after which they were kept at room temperature for the remainder of the time. The loss on retesting from three years eight months to one year three months later was from 40 to 12.5 per cent.

The second series of tests were on 16 lots of serum in sealed bulbs kept in the ice-chest at 4° C. from three years seven months to two years six months. The maximum loss was 16.66 per cent, half of the number showing no loss.

Seven lots that had been on the market and were returned after the expiration of the time limit were tested and found to have lost from 16.7 per cent to no loss in two lots. Of seven lots kept in bulk in the ice-chest from 10 months to 16 months, only one showed loss.

Kinyoun and Hitchens[6] tested the loss in strength of 100 lots of serum that had been returned after being on the market and kept under unknown conditions from 13 to 27 months. They state that in the original tests the unit strength was determined within 50 units, but that on the retest within 10 units. It was found that the loss in

[1] *Centralbl. f. Bakt.*, Orig., 1898, 23, p. 934.

[2] *Festschrift von Robert Koch*, 1903, pp. 451–62.

[3] "Diphtheria Antitoxin" (editorial), *Jour. Roy. Army Med. Corps*, 1904, 2, pp. 601–2.

[4] *Centralbl. f. Bakt.*, Orig., 1905, 38, pp. 233–36.

[5] *Amer. Med.*, 1905, 10, pp. 746–48.

[6] *Centralbl. f. Bakt.*, Ref., 1907, 40.

unit value ranged from 0 to 48.6 per cent, and that at least 65 per cent of the packages, irrespective of the unit strength, showed a depreciation in value of 25 per cent for each 12 to 15 months. They conclude that an excess of 24 per cent should be added to all serum on the market to allow for deterioration in potency. This study is of particular value on account of it having been made on a large number of samples kept under commercial conditions.

METHOD.

The serum was furnished by the contributors either in small vials containing about 3 c.c. each or in the usual "ready-to-use" syringe in which antitoxin is commonly marketed in the United States. In this way an unopened package was used for each successive test.

As soon as possible after the serum was received at the laboratory its potency was determined within 10 units. The entire lot of serum was then divided into three portions: One portion was kept at room temperature, one in the cold room at 15° C., and the third in the ice-chest at 5° C., all three portions, of course, being kept in the dark.

Every six months after the beginning of the experiment the potency of each portion was again determined within 10 units. All tests subsequent to the first were so made that the highest potency for which it was tested was the strength determined at the previous test. In this way there was always shown in each test the decrease in potency since the previous one and an approximate indication was had of the probable decrease for the ensuing six months. In every instance two guinea-pigs were placed on each dose, in order that any irregularities might be indicated and avoided. All the guinea-pigs used were of standard weight, between 250 and 280 gm., and were from the stock raised at the laboratory and known to have no inherited immunity to diphtheria toxin. At least two and usually three controls were given the L+ or test dose of toxin used against one standard unit, and if an irregularity determined by the death of the controls was noted the test was repeated.

An effort was always made to place the lower limit of tests so that some of the animals that failed to die an acute death would develop paralysis, in order that data might be obtained as to the ratio between the least amount of antitoxin conferring complete protection and that permitting the development of paralysis.

On account of the large number of guinea-pigs required for this work, some 4,000 altogether, serious inroads were made upon our

stock of guinea-pigs; but it was felt that the importance of the work fully justified their use.

All dilutions and inoculations were made by me individually, so that any personal factors that might have entered into the work were largely eliminated.

The following charts show the results of the tests of 18 lots of serum in semi-annual periods when kept at 5° C., 15° C., and room temperature.

The serum kept at room temperature was in the large bacteriological laboratory room. In the winter the temperature of this room was about 70° F.; in the summer it varied with the diurnal weather conditions of Washington, D.C., sometimes reaching a maximum temperature of 95° to 100° F.

The legend at the top of each chart shows the age of the serum when the experiment was begun, potency at that time, and the kind and amount of preservative used.

The three curves on each chart show the percentage of loss at each successive observation from the original strength, and the actual strength at the last test.

Charts 1 to 14 give the results obtained in the testing of untreated antitoxic serum; Charts 15 to 18, results with serum precipitated and concentrated by the Gibson process.

THE KEEPING QUALITIES OF DRIED ANTITOXIN.

It has been shown by others as well as by me that diphtheria antitoxin in the liquid serum inevitably loses in potency by age, but there does not seem to have been any work reported as to the keeping qualities of antitoxin in the dried state. In May, 1904, I prepared a large amount of dried diphtheria antitoxin to be used in the preparation of the American standard diphtheria unit. Most of this serum was kept in the special bulb that I designed for this purpose,[1] under vacuum and P_2O_5. A few bulbs were kept without the acid and not under vacuum. The dried serum at the time of its preparation in 1904 contained 5,250 units per gm. Recently I tested the potency of some of the serum kept since 1904 at 5° C., but not under vacuum

[1] M. J. Rosenau, "The Immunity Unit for Standardizing Diphtheria Antitoxin," *Bull. 21, Hygienic Laboratory*, February, 1905.

and P_2O_5, and found it still to contain 5,250 units per gm.; in other words, the dried serum in $5\frac{1}{2}$ years is of the same potency as when first prepared. It is to be regretted that some of the same serum was not placed at room temperature and at 15° C. in order to determine if it retains its potency as well under these conditions as at 5° C. I am inclined to believe that such would have been found to be the case, or at most, the loss would have been very slight.

As the dried serum apparently retains its potency with but slight impairment, it would seem that dried diphtheria antitoxin is peculiarly suited for shipment to the tropics, or for use on ships that make long voyages. The only drawback to its use appears to be in the making of a sterile solution, and this could largely be overcome by aseptic precautions by the user and the producer of the serum.

SUMMARY AND DISCUSSION.

A study of the preceding charts shows that the average loss in percentage strength of the 14 untreated sera for three years at room temperature was 44.2 per cent; at 15° C., 24.4 per cent; at 5° C., 16.7 per cent.

The average percentage loss in potency of the same sera for two years at room temperature was 32.8 per cent; at 15° C., 18.5 per cent; at 5° C., 12.4 per cent.

The average percentage loss for one year at room temperature was 18.7 per cent; at 15° C., 10.2 per cent; at 5° C., 6.7 per cent.

The average loss in percentage strength of the 4 sera concentrated by the Gibson method for two years at room temperature was 25.8 per cent; at 15° C., 18.8 per cent; at 5° C., 12.9 per cent.

The average percentage loss of the same sera for one year at room temperature was 16.4 per cent; at 15° C., 10.5 per cent; at 5° C., 8.8 per cent.

From the above it will be seen that there was but little difference in the keeping qualities of the untreated serum and that concentrated by the Gibson process except for the serum kept at room temperature for two years, in which case there was a difference in favor of the Gibson serum of about 8 per cent. It would seem that the yearly loss in potency is about 20 per cent, altho occasionally it may go as high

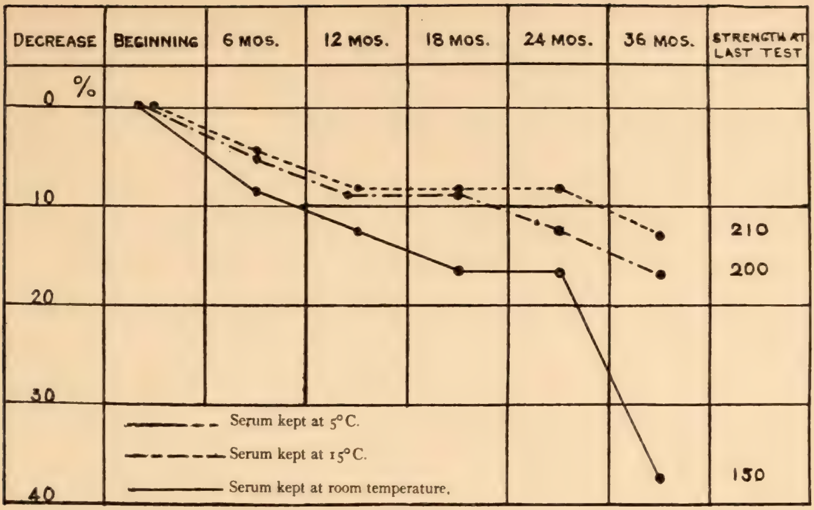

CHART 1.—Serum No. 1,110, F.S. Blood drawn August 29, 1904. Serum preserved with 0.4 per cent trikresol. 549 days from collection of serum to beginning of experiment. Strength when experiment was begun, 240 units per c.c.

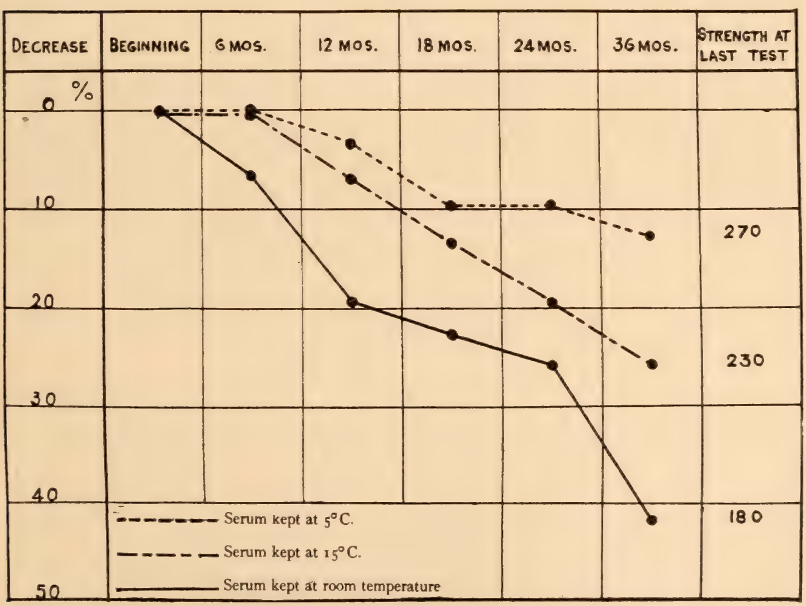

CHART 2.—Serum No. 08034, P.D. Blood drawn September 16, 1905. Serum preserved with 0.4 per cent trikresol. 70 days from collection of serum to beginning of experiment. Strength when experiment was begun, 310 units per c.c.

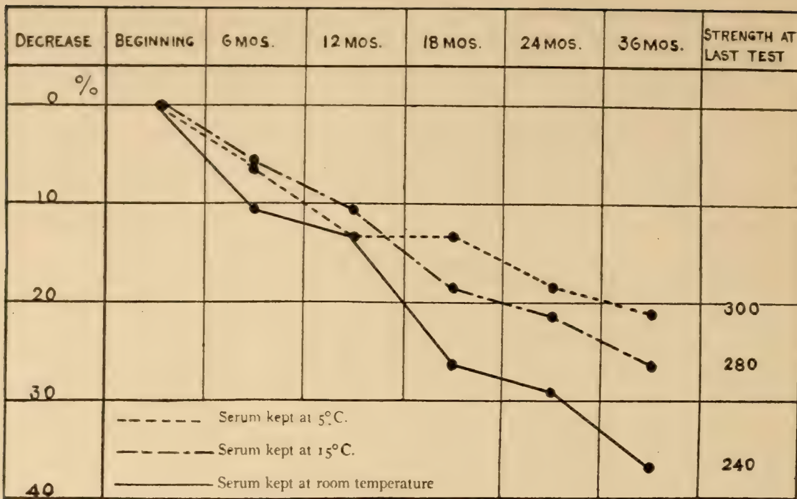

CHART 3.—Serum No. 192, H.M.A. Blood drawn August 14, 1905. Serum preserved with 0.4 per cent trikresol. 191 days from collection of serum to beginning of experiment. Strength when experiment was begun, 380 units per c.c.

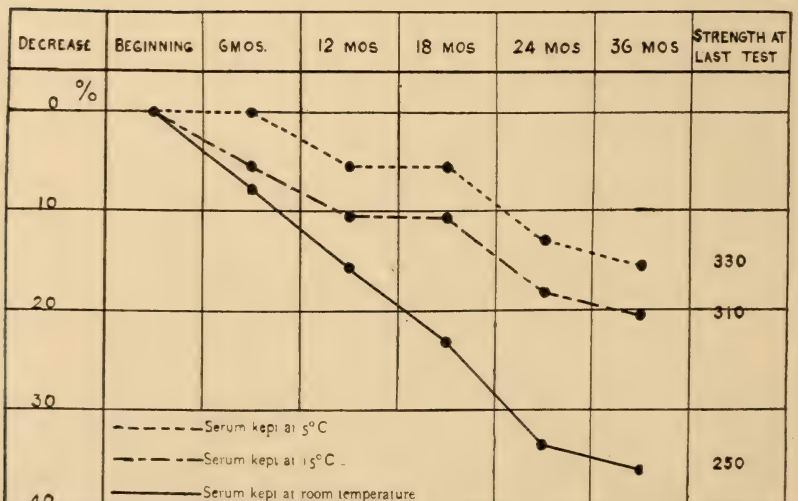

CHART 4.—Serum No. 08033, P.D. Blood drawn September 16, 1905. Serum preserved with 0.4 per cent trikresol. 80 days from collection of serum to beginning of experiment. Strength when experiment was begun, 390 units per c.c.

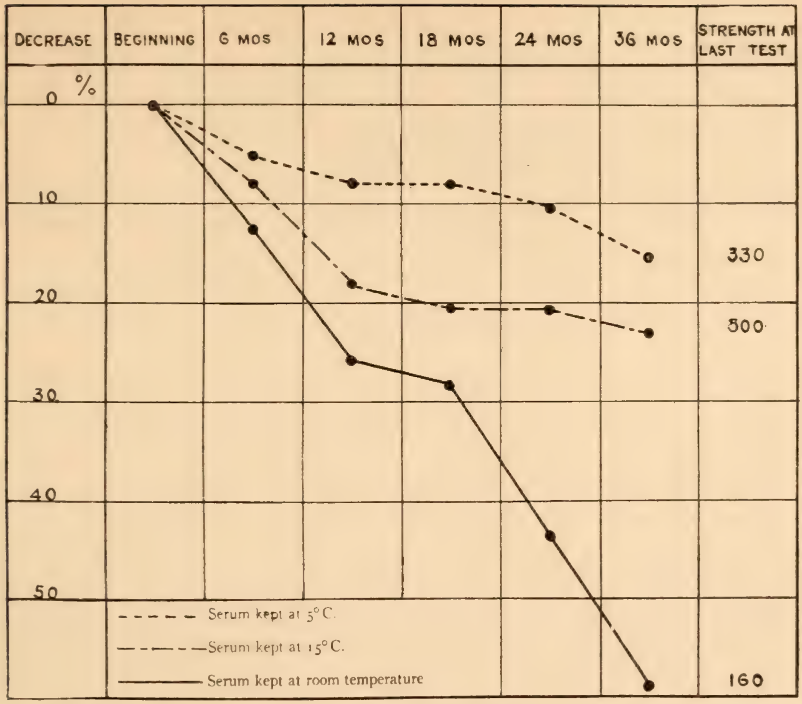

CHART 5.—Serum No. 08023, P.D. Blood drawn September 17, 1905. Serum preserved with 0.4 per cent trikresol. 151 days from collection of serum to beginning of experiment. Strength when experiment was begun, 390 units per c.c.

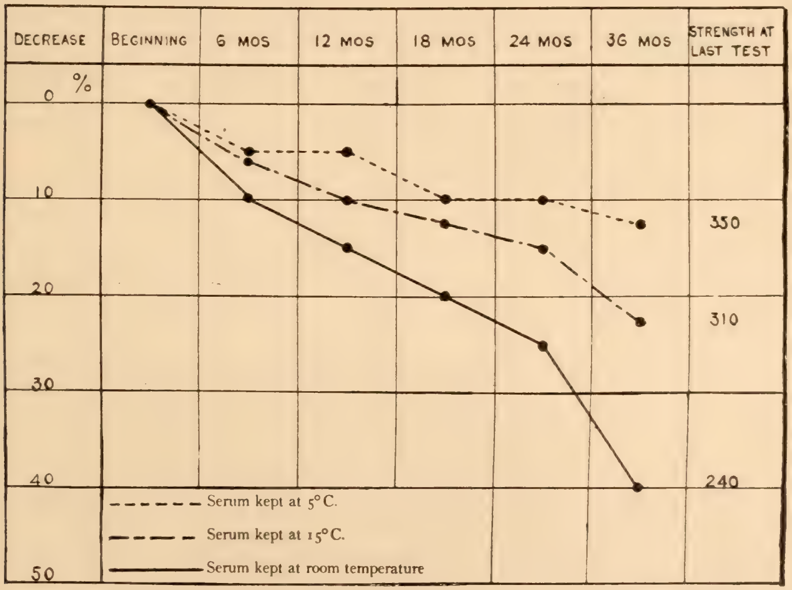

CHART 6.—Serum No. 1,351, F.S. Blood drawn February 13, 1905. Serum preserved with 0.4 per cent trikresol. 385 days from collection of serum to beginning of experiment. Strength when experiment was begun, 400 units per c.c.

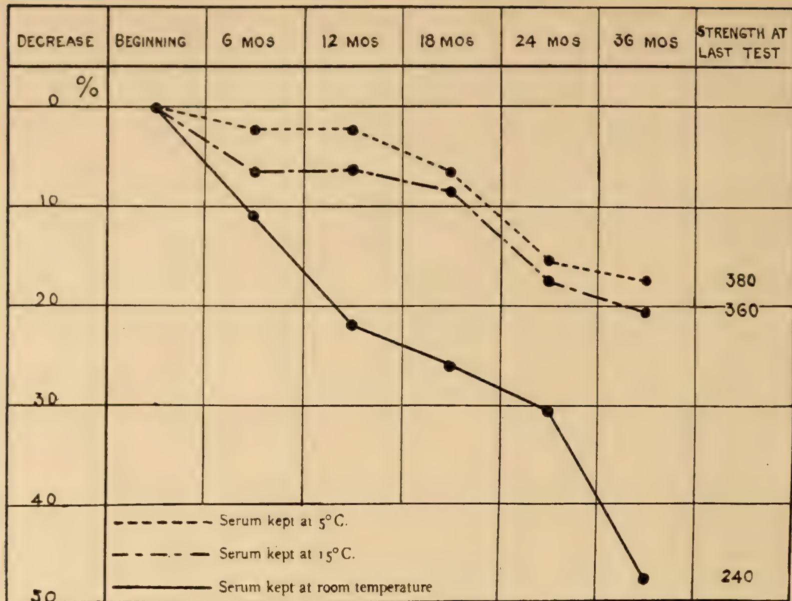

CHART 7.—Serum No. 08021, P.D. Blood drawn September 17, 1905. Serum preserved with 0.4 per cent trikresol. 101 days from collection of serum to beginning of experiment. Strength when experiment was begun, 460 units per c.c.

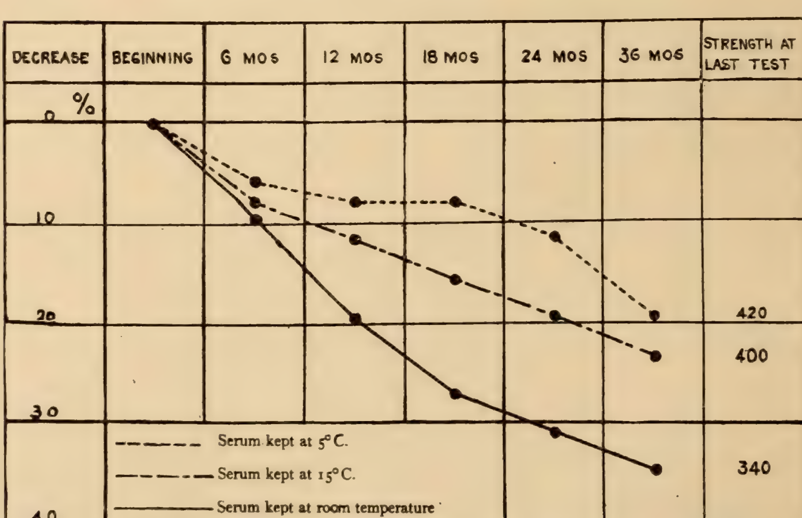

CHART 8.—Serum No. 1,429, F.S. Blood drawn May 8, 1905. Serum preserved with 0.4 per cent trikresol. 253 days from collection of sérum to beginning of experiment. Strength when experiment was begun, 520 units per c.c.

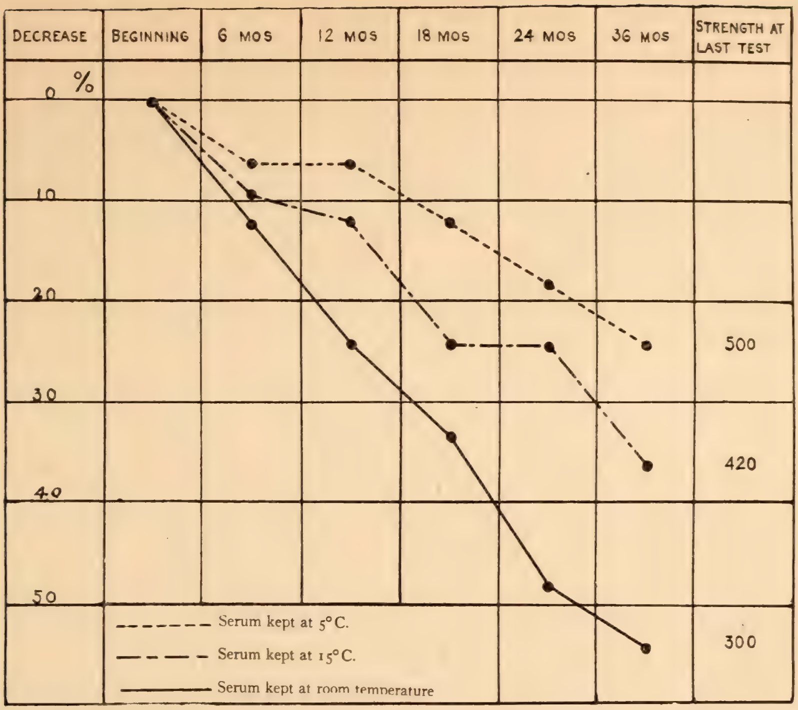

CHART 9.—Serum No. 07635, P.D. Blood drawn May 1, 1905. Serum preserved with 0.4 per cent trikresol. 240 days from collection of serum to beginning of experiment. Strength when experiment was begun, 660 units per c.c.

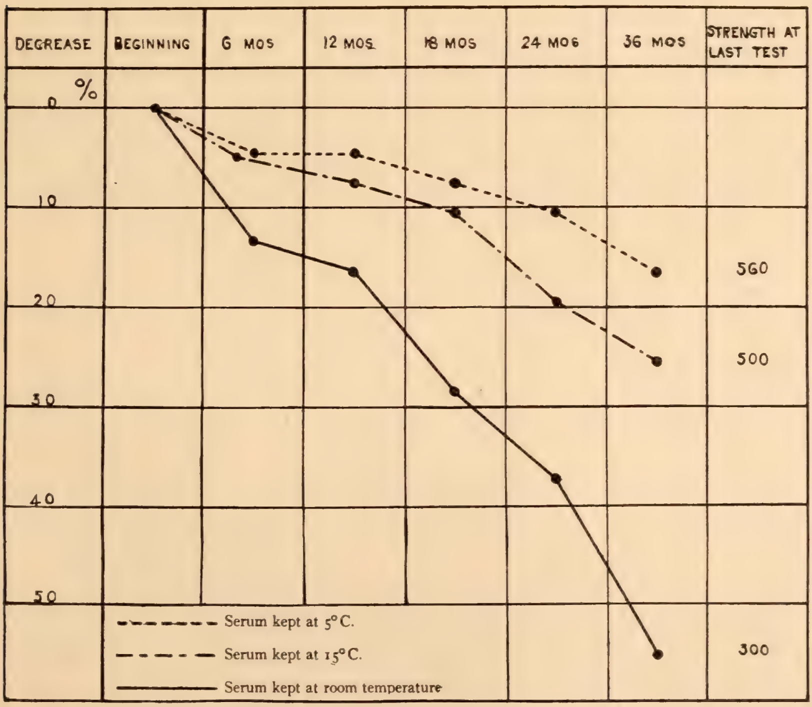

CHART 10.—Serum No. 08022, P.D. Blood drawn September 17, 1905. Serum preserved with 0.4 per cent trikresol. 100 days from collection of serum to beginning of experiment. Strength when experiment was begun, 670 units per c.c.

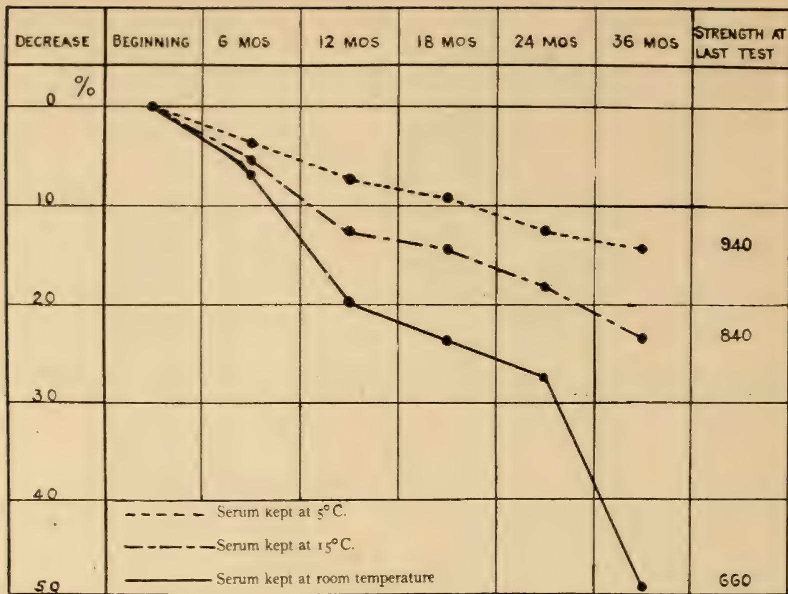

CHART 11.—Serum No. 1,500, F.S.Co. Blood drawn August 21, 1905. Serum preserved with 0.4 per cent trikresol. 374 days from collection of serum to beginning of experiment. Strength when experiment was begun, 1,100 units per c.c.

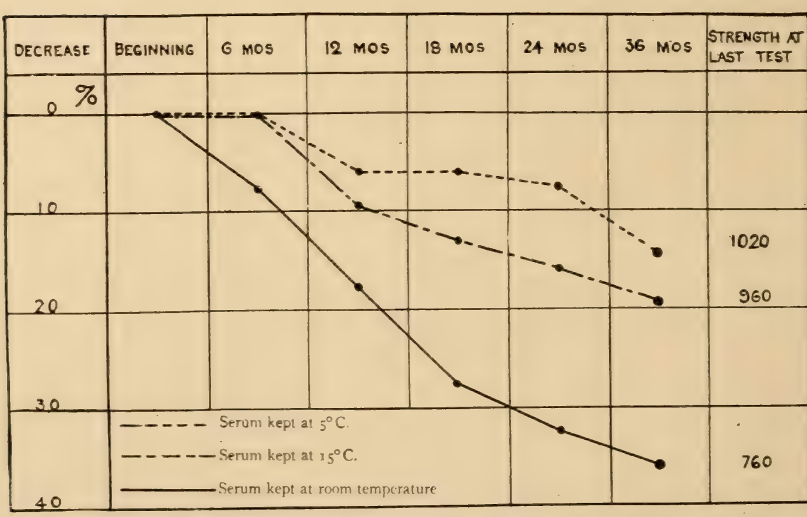

CHART 12.—Serum No. 305, N.Y.H.D. Blood drawn May 18, 1906. Serum preserved with chloroform. 20 days from collection of serum to beginning of experiment. Strength when experiment was begun, 1,190 units per c.c.

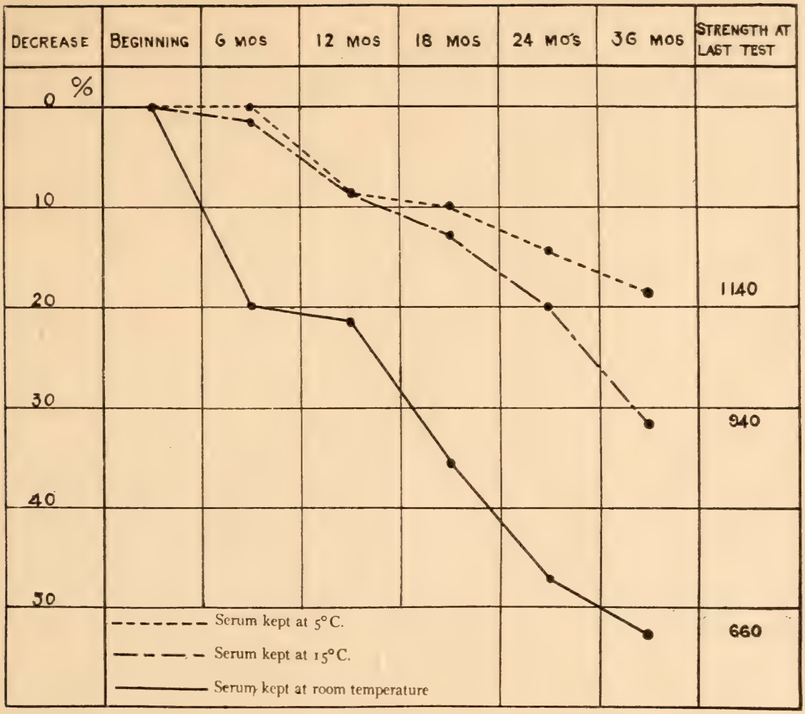

CHART 13.—Serum No. 306, N.Y.H.D. Blood drawn May 18, 1906. Serum preserved with chloroform. 20 days from collection of serum to beginning of experiment. Strength when experiment was begun, 1,400 units per c.c.

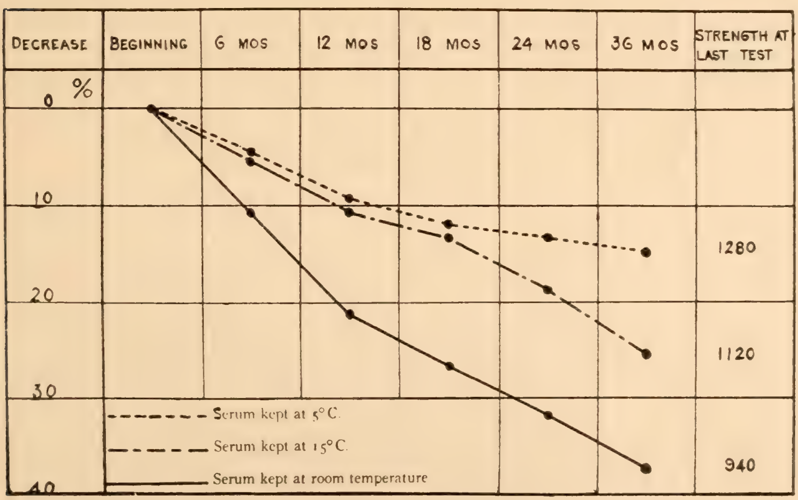

CHART 14.—Serum No. 310, N.Y.H.D. Blood drawn June 4, 1906. Serum preserved with chloroform. 88 days from collection of serum to beginning of experiment. Strength when experiment was begun, 1,500 units per c.c.

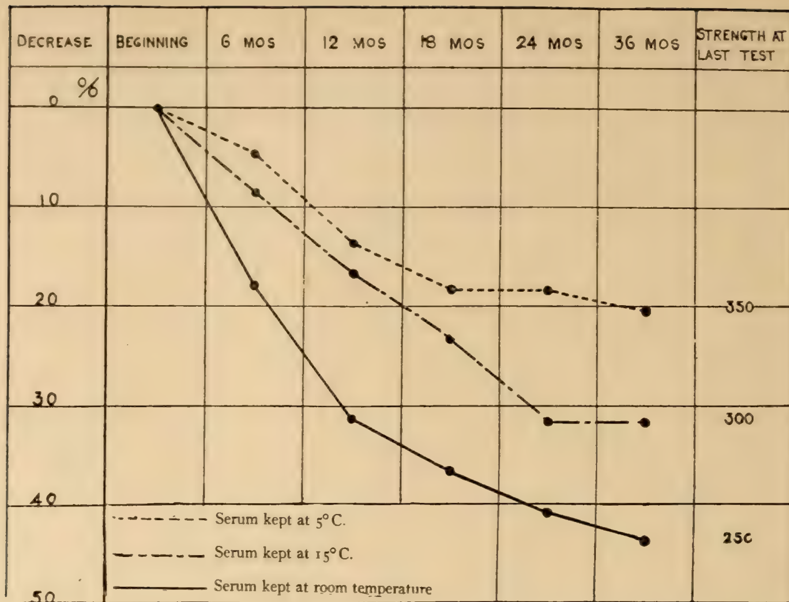

Chart 15.—Serum No. 245, H.M.A. Concentrated after Gibson's method. Concentration completed November 30, 1906. Preserved with chloroform. 35 days from concentration of serum to beginning of experiment. Strength when experiment was begun, 440 units per c.c.

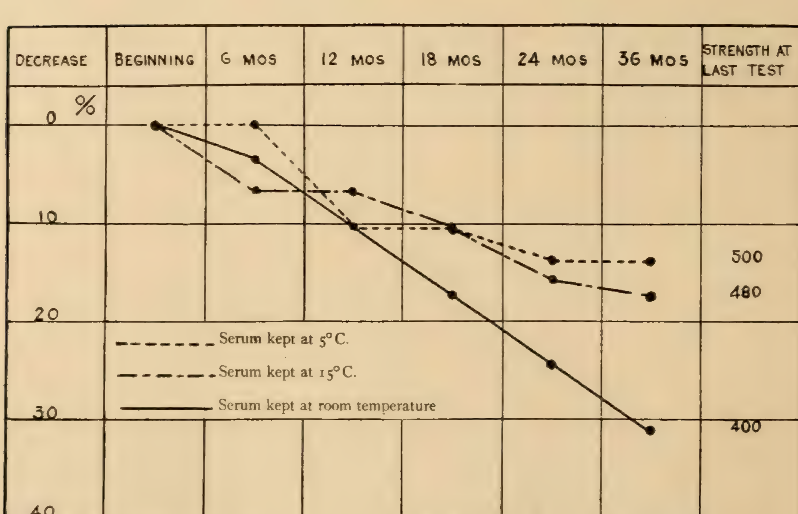

Chart 16.—Serum No. 10B, N.Y.H.D. Concentrated after Gibson's method. Preserved with chloroform. 22 days from concentration of serum to beginning of experiment. Strength when experiment was begun, 580 units per c.c.

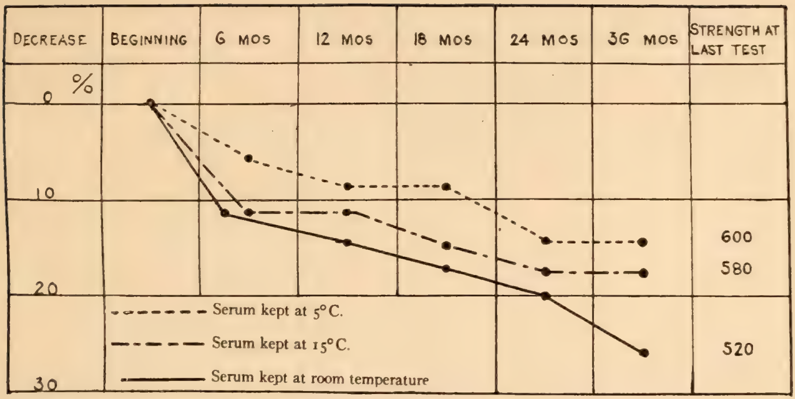

CHART 17.—Serum No. 13C, N.Y.H.D. Concentrated after Gibson's method. Concentration completed February 26, 1909. Preserved with chloroform. 26 days from concentration to beginning of experiment. Strength when experiment was begun, 700 units per c.c.

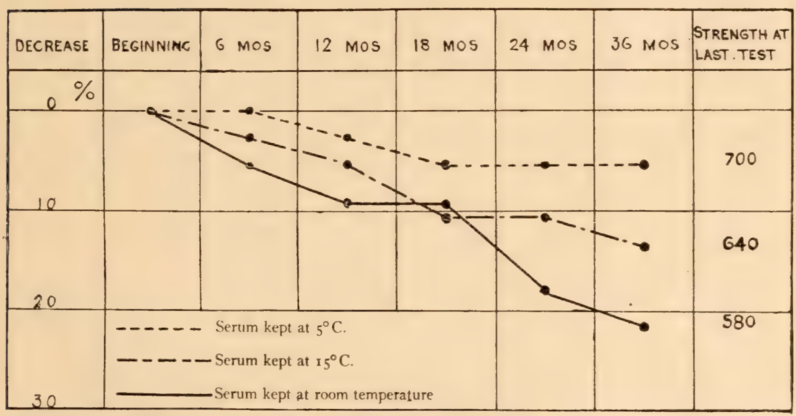

CHART 18.—Serum A249, H.M.A. Concentrated after Gibson's method. Concentration completed December 29, 1906. Preserved with chloroform. 17 days from concentration of serum to beginning of experiment. Strength at beginning of experiment, 740 units per cubic centimeter.

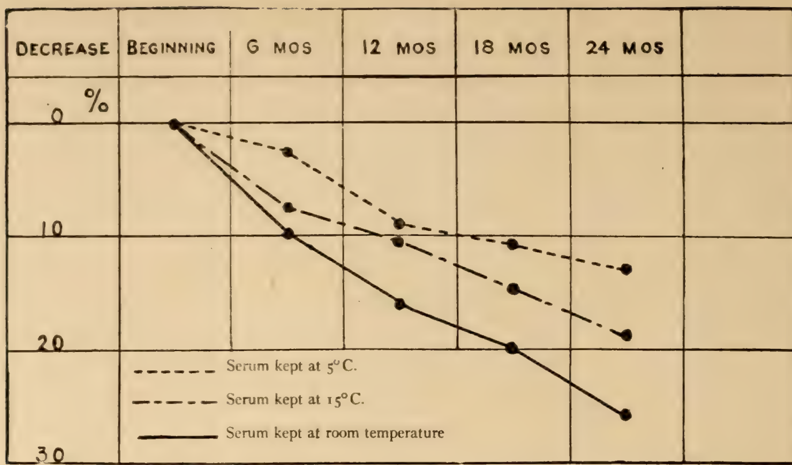

CHART 19.—Showing the average decrease in potency of 14 lots of antitoxic horse serum when kept at 5° C., 15° C., and room temperature, respectively.

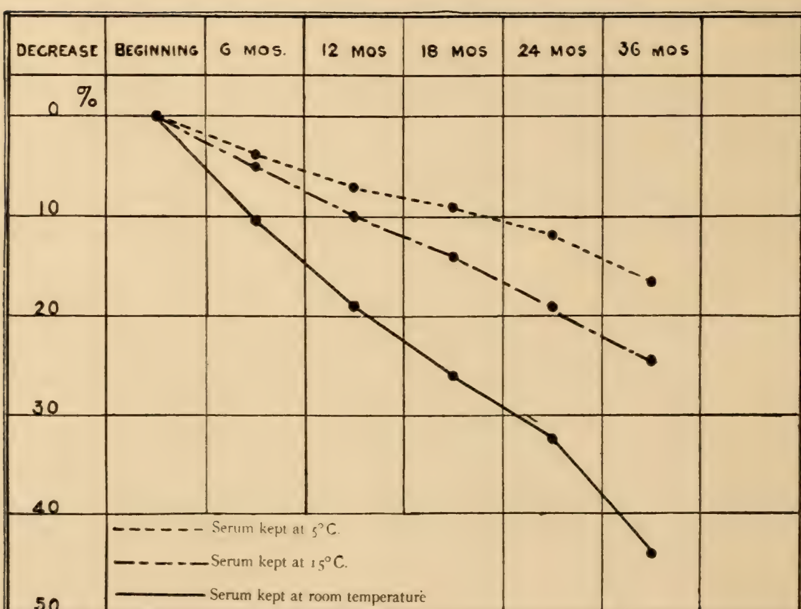

CHART 20.—Showing the average decrease in potency of 4 lots of antitoxic horse serum concentrated by the Gibson process, and kept at 5° C., 15° C., and room temperature, respectively.

as 25 per cent when the serum is kept at room temperature. When kept at 15° C., the average yearly loss is about 10 per cent, altho it may sometimes be double that, but this is exceptional. At 5° C., the average yearly loss falls to about 6 per cent, but there may be instances in which the loss is considerably greater.

The maximum loss of any serum in three years at room temperature was 59 per cent; at 15° C., 36.4 per cent; at 5° C., 24 per cent; the last two being in the same serum.

The minimum loss for three years at room temperature was 34.6 per cent; at 15° C., 16.7 per cent; and at 5° C., 12.5 per cent; the last two being in the same serum.

One of the very interesting points that were brought out by the experiments was as to the relation of the antitoxic content of a serum to its therapeutic value. It was found that in the test mixtures the protective value of the serum was in exact accord with its unit value; for example, 1/1,500 c.c. of Serum 310 was of the same protective value as 1/150 c.c. of Serum 1,110; the first serum contained 1,500 units per c.c. and the last only 150 per c.c. There can be no question as to the protective value of the serum being in direct relation to its unit value for, in our 200 determinations of the antitoxic potency of serum in this study, the same result was had in each instance.

It was noticed that all sera, even of the same age and kept under the same conditions, did not deteriorate in the same ratio; just why this should be so I am unable to decide, but it is apparently independent of external influences and probably depends on some inherent property of the serum.

The addition of preservatives, such as chloroform and trikresol, apparently did not exert any influence upon the deterioration of the antitoxin as there was no appreciable difference in the behavior of the sera preserved with these two substances.

The opinion held by some physicians that old sera or sera on which the return dates have expired should not be used is without scientific basis, and the only reason for not using such sera would be that a larger amount would have to be given to make up for the decrease in potency. Old sera, unit for unit, are just as potent as fresh sera, and would be, perhaps, less apt to cause severe serum reactions than fresh sera.

CONCLUSIONS.

The average yearly loss in potency of diphtheria antitoxin at room temperature is about 20 per cent; at 15° C., about 10 per cent; at 5° C., about 6 per cent, altho in some instances these percentages may be much increased.

As a result of this work, there appears to be but little difference in the keeping qualities of untreated sera and sera concentrated by the Gibson process.

Diphtheria antitoxin to be placed upon the market and there kept under unknown conditions as regards temperature should not be labeled with a return date longer than two years and should contain an excess of at least 33 per cent to allow for decrease in potency; in addition, when the serum is sold in syringes with an absorbable piston, an excess should be added for this loss.

Dried diphtheria antitoxin kept in the dark, at 5° C., retains its potency practically unimpaired for at least 5½ years.

The lack of confidence in the therapeutic properties of old sera is without basis, as such sera, unit for unit, are as potent as new sera.

The protective value of diphtheria antitoxin is in exact accord with its unit value, and is independent of the volume of the serum or other properties in the serum.

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ON THE MILLS-REINCKE PHENOMENON AND HAZEN'S THEOREM CONCERNING THE DECREASE IN MORTALITY FROM DISEASES OTHER THAN TYPHOID FEVER FOLLOWING THE PURIFICATION OF PUBLIC WATER-SUPPLIES.*

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I. INTRODUCTION.

IT is nowadays commonly understood that the purification of a polluted water-supply produces a marked decrease in the mortality from typhoid fever among persons using the water for drinking and other domestic purposes, but it is not as yet generally recognized that such purification produces also a marked decrease in deaths from other diseases.

In 1893–94 it was observed, independently, by Messrs. Hiram F. Mills, C.E., of Lawrence, Massachusetts, and Dr. J. J. Reincke, of Hamburg, Germany, that the purification of the polluted public water-supplies of Lawrence and of Hamburg, respectively, was producing a notable decline in the general death-rate of each of these cities. The attention of Mr. Allen Hazen was about the same time turned to the subject, and some years later, in a paper presented to the International Engineering Congress held at the St. Louis Exposition in 1904, he drew from an examination of the death-rates of certain cities which had radically improved polluted water-supplies the following conclusion:

> Where one death from typhoid fever has been avoided by the use of better water, a certain number of deaths, probably two or three, from other causes have been avoided.

This novel statement has not hitherto received the attention which it deserves, and in view of this fact, as well as in order to test

the range and accuracy of Mr. Hazen's conclusion, and also if possible to find an explanation for it, we have undertaken a careful examination of the vital statistics of a number of cities each of which has changed more or less suddenly from a polluted to a purified water-supply.

On August 14, 1908, we published in "Science" a preliminary communication announcing in part the results of our study and confirming Mr. Hazen's statement. We also contributed to the volume on "Tuberculosis in Massachusetts," prepared by the Massachusetts State Committee for the International Congress on Tuberculosis held in Washington, D.C., September 21 to October 12, 1908, and published in Boston in 1908, a paper entitled "On an Apparent Connection between Polluted Public Water-Supplies and the Mortality from Pulmonary Tuberculosis."

As will appear beyond, circumstances arose not long after the publication of our preliminary communication which made it seem desirable to repeat some portions of our work, and especially to take into consideration questions relating to the constancy of increase of population ordinarily estimated for American cities between census years. Some delay has thus been caused, but we now present the complete paper, which we venture to believe has meanwhile gained both in scope and in trustworthiness.

II. THE MILLS-REINCKE PHENOMENON.

Shortly after the introduction of a filtered and purified water-supply into Lawrence, Massachusetts, in September, 1893, it was observed by Mr. Hiram F. Mills, C.E., a member of the State Board of Health of Massachusetts, then a resident of the city of Lawrence and chief engineer of the company controlling the water power of that city, that a marked decrease in the general death-rate of the city, and not merely in the death-rate from typhoid fever, was taking place. A few months earlier (May, 1893) filtration of the public water-supply had likewise been established for the city of Hamburg, Germany, and there also it was observed by Dr. J. J. Reincke, health officer of that city, that the general death-rate was declining more rapidly than could possibly be accounted for by the deaths from typhoid fever alone. To this important discovery, made thus inde-

pendently by Mr. Mills in Lawrence and Dr. Reincke in Hamburg, we have, because of its fundamental and far-reaching significance, applied the name of *The Mills-Reincke Phenomenon.*

Mr. Allen Hazen, the now well-known civil and sanitary engineer, who had long been more or less closely associated with Mr. Mills, visited Europe early in 1894, and while in Hamburg conferred with Dr. Reincke. He was impressed with the favorable results of filtration as observed and described by Dr. Reincke, and on his return to America he mentioned Dr. Reincke's observations to one of the writers (W. T. S.). He also stated in the first edition (1895) of his "Filtration of Public Water-Supplies" (p. 177):

> The death-rate [of Hamburg] since the introduction of filtered water has been lower than ever before in the history of the city, but as it is thought that other conditions may help to this result no conclusions are as yet drawn.

To this statement he appended in a later edition a table showing the deaths in Hamburg from typhoid fever and from all causes 1880–98, i.e., before and after the introduction of filtration.

III. DR. REINCKE'S OBSERVATIONS ON THE RELATION OF TOTAL DEATH-RATE TO WATER-SUPPLY IN HAMBURG, GERMANY, 1894–97.

The Hamburg filters began their work in May, 1893, and for that year the total death-rate showed an extraordinary decrease from that of preceding years, and a greater decrease than could be accounted for by the drop in the typhoid fever death-rate alone. Dr. Reincke's observations concerning this remarkable phenomenon were published from time to time in his Annual Reports[1] and may be quoted (in literal translation) or summarized as follows:

> In the year 1893 . . . . the total death-rate of Hamburg was 20.4 per thousand living. The mortality has never been so low before . . . . since the beginning of vital statistics in Hamburg in 1820. . . . . Manifestly the chief explanation of this favorable result is that the cholera of 1892 carried off an extraordinarily large number of people, especially of the weaker and less resistant, a part of whom would otherwise not have died until 1893. Whether the great improvement of the water has been of influence can be ascertained only after several years. The figures given in connection with infant mortality speak, however, strongly for this theory (Report for 1893, p. 15).

[1] *Berichte des Medizinal-Inspektorats über die medizinische Statistik des Hamburgischen Staates.*

For 1894 Dr. Reincke found the total death-rate to be 17.9 per thousand living.

Thus the mortality has receded still lower even than that of last year, which, as has been said, was the most favorable since the beginning of recorded vital statistics in Hamburg. . . . .

It is not altogether easy to offer a sufficient explanation of this favorable result. That the effect of the cholera of 1892 in carrying off prematurely many weak individuals is still operative, is not very probable, for the favorable conditions of 1894 appear in no age group so strikingly as among infants under one year, all of whom have been born since the epidemic. The death-rate of such infants is 201.9 per thousand [under one year of age] for 1894, as compared with a fifteen-year average of 287.1.

On the other hand, there is greater reason than in the preceding year for attributing a considerable share in the altered conditions to the improved water-supply, and the results for 1892 and 1893 of the influence of the water on the mortality of children from diarrhea and gastro-intestinal diseases are freshly confirmed (Report for 1894, pp. 11, 13).

In 1895 the total death-rate was 18.9 per thousand living, i.e., 1.0 more than in 1894.

Nevertheless the mortality was still more favorable by 2.7 per thousand than in 1890, the most favorable year before the introduction of filtration, and more favorable by 5.8 than the mean of the ten years before the cholera epidemic, i.e., 1882–91 (Report for 1895, p. 11).

For 1896 the total death-rate is reckoned at 17.1 per thousand living, the lowest ever observed in Hamburg up to that time.

Undoubtedly, the especially favorable result for this year is to be attributed chiefly to the cool wet weather from the middle of June to the middle of July, on account of which the high infant mortality of the summer was speedily cut down. . . . . That the year has passed as favorably as the three preceding years is fresh confirmation of the fact that *since the filtration of the drinking water the health conditions in Hamburg have improved in a quite extraordinary manner.* (Ist eine neue Bestätigung dafür, dass seit der Filtration des Trinkwassers sich die Gesundheitsverhältnisse Hamburgs in ganz ausserordentlicher Weise verbessert haben.) (Report for 1896, p. 11, italics ours.)

For 1897 the total death-rate of Hamburg "Stadt" (exclusive of the suburbs) is reckoned as 17.0 per thousand living. Again no rate so low had ever before been observed in the history of the city (Report for 1897, p. 12).

All the more weight must be given to the statistical statements made above because these were based upon an annual official enumeration of the population.

IV. DR. REINCKE'S STUDIES ON THE RELATION OF INFANT MORTALITY TO WATER-SUPPLY IN HAMBURG AND ALTONA, 1893–97.

In Dr. Reincke's Annual Report for 1892[1] we find a searching examination of the effect of water-supply upon infant mortality (particularly as due to gastro-intestinal diseases), which, b cause of its early date and its obvious importance for all students of the relations of polluted water to public health science, we shall either quote in careful translation or summarize, as follows:

It is usually assumed that the greater part of the deaths from gastro-intestinal diseases (*Brechdurchfall*)[2] in summer is to be explained by the high summer temperature, particularly through the action of warmth on the principal food of infants, i.e. milk. Our local observations, however, indicate that the matter is somewhat more complicated than this (p. 10).

Tables and diagrams are given of deaths from diarrhea and gastro-intestinal diseases, by months, for Hamburg and for Altona, with the mean monthly temperatures, 1871–92. In connection with these it is pointed out that the parallelism between deaths and high temperatures is not well marked, but that, on the contrary, there have been distinct outbreaks of these diseases during cold-weather periods. Also, that the curves for winter deaths of infants under one year from gastro-intestinal diseases are not parallel in Hamburg and Altona, as is the case with those of the summer period Reincke was unable to discover similar winter outbreaks in any other German city except, for certain years, in Berlin. We quote further and at length:

In Bockendahl's "Generalbericht über das öffentliche Gesundheitswesen der Provinz Schleswig-Holstein für das Jahr 1870," p. 10, we read: "Still more striking was an epidemic of gastro-intestinal diseases in Altona in January which proved fatal to 43 children. As the cases were observed in all parts of the city and the medical officer was unable to explain the phenomenon, he procured from the Gas and Water Company a statement regarding any interruptions in the supply of pure water to the city during the year 1870. He then learned that the city was supplied during a few days in January with unpurified Elbe River water. However little reason there may

[1] *Bericht des Medizinal-Inspektorats über die medizinische Statistik des Hamburgischen Staates für 1892*, published without date, but presumably late in 1893.

[2] We have not been able to find any exact English equivalent for the term *Brechdurchfall*, which denotes literally a condition characterized by diarrhea and vomiting. Mr. Hazen's rendition "cholera infantum," employed in his translations from Dr. Reincke in Appendix II of his "Filtration of Public Water-Supplies," does not appear to be allowable. *Brechdurchfall* is applied to both adults and children, but more frequently to the latter because gastro-intestinal disturbances are much more common during the early years of life. Throughout our translations of, and references to, the writings of Dr. Reincke and his associates, we shall render the term, with an approximation to accuracy, sometimes as "gastro-intestinal disorders," and sometimes, especially when it is applied to fatal cases, as "gastro-intestina diseases," without, however, necessarily implying by the latter phrase the existence of discoverable lesions.

be for regarding the connection between these two circumstances as proved, it is only proper that at all times interruptions in the pure water service be closely watched, because only in this way can reliable conclusions be reached and dangers be avoided. . . . ."

Dr. Kraus, who was at that time the medical officer of Altona, and who made the studies upon which Bockendahl's statement is based, subsequently became health officer of Hamburg; and, in a more lengthy opinion . . . . dated 1874, earnestly advocating the establishment of sand filtration in Hamburg, supported his position with the above observations, although with the caution that they are not to be regarded as entirely conclusive. In his annual report on the vital statistics of Hamburg for 1875 he suggests (p. 17) that the addition of unfiltered Elbe water to milk had probably been connected with the infant mortality of Hamburg, which mortality was very high in comparison with that of London (dass der Zusatz von unfiltrirtem Elbewasser zu der Milch wahrscheinlich an der im Vergleich zu London sehr hohen Sauglingssterblichkeit Hamburgs betheiligt sei.) He later repeated this opinion both in oral and in written statements.

Meantime so much material for observation has accumulated that his view may now fairly be regarded as proved. . . . .

Years ago, on observing the epidemic behavior of typhoid fever, in Altona . . . . I suggested that at the Altona waterworks disturbances in the action of the filters occurred in connection with frost, causing the distribution of water insufficiently purified. Wallichs of Altona, on the ground of further observations on typhoid fever, has come to the same conclusion, and lately Robert Koch, on the occasion of the small winter epidemic of Asiatic cholera in Altona, has proved that our hypothesis is correct. When open filters are cleaned in cold weather, it is a matter of common observation that the germs in the water are not sufficiently held back by the filters; and, as an actual fact, simultaneously with these disturbances of filtration there have occurred not only the explosive typhoid outbreaks of 1886, 1887, 1888, 1891, and 1892, and the Asiatic cholera outbreaks of 1871 and 1893, but also the increases of gastro-intestinal disorders in Altona. It can, therefore, no longer be doubted that these phenomena stand in the relation of cause and effect. It is thus explained also why in warm winters, as above mentioned, there have been no such outbreaks, and why gastro-intestinal disorders in winter have not been parallel in Hamburg and Altona.

In Hamburg the winter increases [of gastro-intestinal disorders] cannot be explained so simply. It seems probable, rather, that northeast storms, through the high tides which they cause, bring polluted water in greater quantity to the intake of the waterworks. It cannot be demonstrated that every such occurrence has caused an increase in infant mortality, but in many instances a noteworthy coincidence occurs. . . . . This point could be decided only by careful bacteriological determinations, and we have some such evidence for the last months of the year 1892, when daily bacteriological examinations of the city water were made at the Hygienic Institute, altho examinations of the river water itself were not begun until later. It was thus found that the bacterial content of the water varied between 200 and 600 at the end of October and the beginning of November; that it rose after November 19 to about 3,000; after December 4, to 4,000–5,000; and on December 18, to 7,500; and finally dropped rapidly in January to 200–500. On December 4 there occurred a storm-tide 2.6 m. in height, following a series of moderate high tides, and the December increase in infant mortality corresponded with the increase in bacteria in the water-supply mentioned above.

[TABLE 1.]

Stralau Waterworks, Bacteria per c.c.			Deaths from Acute Intestinal Diseases		
			Total	Gastro-intestinal Diseases	
				At All Ages	Infants under One Year of Age
1886					
March 23	145	March 21–27	...	4	4
March 30	2,300	March 28–April 3	34	20	18
April 6	500	April 4–10	106	49	46
April 13	125	April 11–17	99	46	44
April 20	360	April 18–24	56	25	25
April 27	105	April 25–May 1	46	22	22
1888					
March 1	16	March 11–17	24	4	4
March 15	3,600	March 18–24	76	29	29
		March 25–31	73	25	23
April 3	440	April 1–7	47	17	17
		April 8–14	47	21	21
April 16	125	April 15–21	50	20	18
		April 22–28	27	15	15
		April 29–May 5	28	12	12
1889					
January 16	112	January 13–19	28	4	4
		January 20–26	35	9	8
February 1	1,730	January 27–February 2	38	12	12
		February 3–9	65	17	17
February 15	1,600	February 10–16	86	22	22
		February 17–23	59	13	13
		February 24–March 2	35	8	8
March 4	2,400	March 3–9	47	13	12
		March 10–16	54	22	22
March 18	4,800	March 17–23	112	53	52
		March 24–30	215	118	116
April 1	264	March 31–April 6	120	61	60
		April 7–13	82	40	38
April 15	98	April 14–20	44	18	14
1891					
December 15, 1890.	123	December 21–27, 1890	22	6	6
January 2, 1891	450	December 28, 1890–January 3, 1891	31	4	4
		January 4–10	25	3	3
January 15	1,325	January 11–17	25	6	6
		January 18–24	18	2	2
		January 25–31	36	7	7
February 1	13,000	February 1–7	61	12	12
		February 8–14	158	61	61
February 16	4,000	February 15–21	160	50	50
		February 22–28	104	42	41
March 3	120	March 1–7	88	29	29
		March 8–14	68	20	20
March 17	310	March 15–21	43	14	14
		March 22–28	38	10	10
April 1	85	March 29–April 4	28	9	9
		April 5–11	41	9	9
April 15	800	April 12–18	39	13	13
		April 19–25	46	16	16
May 1	140	April 26–May 2	25	11	11
		May 3–9	49	19	18
1893					
?		February 5–11	23	4	9
?		February 12–18	56	20	19
?		February 19–25	110	41	41
?		February 26–March 4	110	40	39
?		March 5–11	69	20	19
?		March 12–18	39	10	9

A further support for this theory is found in the conditions at Berlin, where, likewise, frost has repeatedly interfered with filtration. The above table gives

the deaths from acute intestinal diseases, for several winter periods in which striking increases in mortalities in these diseases have appeared. It is obvious that, of the deaths enumerated in the first column, a predominant number is among little children. For comparison the bacteriological examinations are given, according to Plagge and Proskauer, of the tap water from the Stralau waterworks. Unfortunately the figures for 1892 are wanting.

No one can doubt, after this demonstration, that here also the delivery of insufficiently purified water every time cost the lives of many children. Still more striking is the evidence, from the publications of the Statistical Office at Berlin, that other parts of the city, supplied with better water from the Tegel works, completely escaped these increases in mortality, precisely as was the case in the well-known typhoid epidemic of February and March, 1889.

. . . . The fatal effects of the water are not confined, however, to those infants dying from well-defined intestinal diseases, for there is a large number besides who must have fallen victims with less striking intestinal symptoms. It appears, furthermore, that almost the only children affected were those not nursed by their mothers or by wet-nurses but fed on the milk of animals or other substitutes, which were mixt with more or less water.

So much for the winter conditions. As for the summer, the work of Plagge and Proskauer shows that the filters have then also occasionally delivered bad water. What influence this may have had is difficult to determine, since at that season gastro-intestinal disorders show ordinarily a high prevalence. But doubtless the influences of weather and water go hand in hand. This hypothesis is still more probable for Hamburg, because there, with the river water diminished during the summer months, the tides easily run up the river [carrying sewage to the intake] even without the aid of storms. This hypothesis is supported by the extraordinary prevalence of gastro-intestinal disorders in the unusually dry warm years of 1886, 1887, and 1892. If this be the case, when the intake is moved higher upstream and sand filtration is started, we may expect a substantial decrease of infant mortality. Not only should the winter increases entirely disappear, but those of the summer ought to be notably diminished. [How well this prophecy was borne out is shown by the table given on p. 121 of the "Gesundheitsverhältnisse Hamburgs im 19. Jahrhundert" published some years later, in 1901.] Moreover, it is not the infants only who will be benefited, for, as the Berlin figures show, there are also many cases of gastro-intestinal disorders among children from one to five years of age, and even adults do not entirely escape (pp. 13–17).

We may mention parenthetically that many of the valuable observations just quoted have been presented in English by Mr. Hazen in the first (1895) and subsequent editions of his "Filtration of Public Water-Supplies," Appendix 11.

Dr. Reincke's report[1] for the following year (1893) gives the mortality under one year of age, reckoned per thousand living in that age-group, for 1893 and earlier years, as follows (p. 17): 1885, 277.2; 1886, 368.8; 1887, 347.5; 1888, 289.8; 1889, 316.5; 1890, 276.3; 1891, 288.7; 1892, 404.8; 1893, **240.2**.

[1] For full title of reports see footnote, p. 492.

How uneven is the distribution by age months of this mortality of infants under one year of age, is shown by the following table.

Economy of space forbids our reproducing in full the table (p. 40) referred to, but we present a summary of it (Table 2).

TABLE 2.

Ages of Infants Dying under One Year, by Months.

	Average 1884–92 Inclusive Percentage	1893 Percentage		Average 1884–92 Inclusive Percentage	1893 Percentage
In the 1st month	24.2	27.0	In the 7th month	5.6	5.7
" " 2d "	12.9	14.3	" " 8th "	5.2	4.5
" " 3d "	11.0	11.9	" " 9th "	4.8	4.2
" " 4th "	9.1	9.6	" " 10th "	4.5	3.8
" " 5th "	7.8	7.2	" " 11th "	4.2	3.0
" " 6th "	6.5	5.9	" " 12th "	4.0	2.9
				100	100

Under "diarrhea and gastro-intestinal diseases" (Durchfall und Brechdurchfall), the following figures are given for the deaths of infants under one year of age for these causes by years:

1884	1,143	1889	1,557
1885	1,189	1890	1,198
1886	1,601	1891	1,500
1887	1,758	1892	2,541
1888	1,063	1893	857

Since I expressed myself in detail on these causes of infant mortality in my report for 1892 [already quoted above], it suffices for the present year [1893] to call attention to the highly significant decline in the number of these deaths and to the fact that this year the increase in mortality in December, often previously observed, is entirely absent. From the thorough studies recorded in last year's report it is highly probable that this favorable result is to be attributed largely to the filtration of the public water-supply, altho this point can be positively decided only after the experience of future years. Especially noteworthy is the considerable increase of deaths from these diseases in the second half of September, following a temporary pollution of the water which led at the same time to an outbreak of Asiatic cholera (pp. 43–44).

Three years later Dr. Reincke repeats a portion of the above table, adding to it the deaths from diarrhea and gastro-intestinal diseases for 1894, 1895, and 1896, which were 708, 918, and 767, respectively (see our Chart 1). He may well have remarked, in view of these figures together with the increasing population:

In no other diseases did the favorable influence of the better water-supply introduced in 1893 stand out so clearly as in these (Report for 1896, p. 46).

V. FURTHER DISCUSSION OF THE INFLUENCE OF WATER-SUPPLY UPON DEATH-RATES IN "DIE GESUNDHEITSVERHÄLTNISSE HAMBURGS IM NEUNZEHNTEN JAHRHUNDERT" (1901).

In 1901 there appeared a comprehensive treatise on the "Sanitary History of Hamburg in the Nineteenth Century," prepared under the editorship of Dr. Reincke.[1] In this work the declines in the total death-rate and in the death-rates from various diseases following the introduction of filtration into Hamburg are still further considered, and additional evidence is given for the existence of a relation between water-supply and gastro-intestinal diseases (particularly of infants). The observations on these points we have abstracted, mainly in literal translations and at some length, as follows:

1. General Death-Rate.

In the century curve of the general death-rate . . . . four peaks of specially high mortality stand out predominantly; these are the cholera years 1831 and 1832, 1848, and 1892 and the small-pox year 1871. Between them lies a greater number of lesser elevations, which are likewise to be attributed to greater or smaller epidemics of infectious diseases. . . . . Following each of these peaks there sets in regularly a compensatory decline in mortality.

Entirely different from these declines is that which set in after the cholera epidemic of 1892; for not only was this deeper than any other before it, but it has also lasted now throughout eight years—the beneficent result of the filtration of the water-supply which has been in operation since 1893 and to which we shall repeatedly return in connection with our consideration of the mortality from particular diseases (p. 90).

Comparing the twenty-five years 1872–1896 with the following four years, the average annual mortality has dropped from 25.0 to 17.2, a decrease of 7.8 per 1,000. In this improvement at least 6.1 per 1,000 may be regarded as due to general measures of sanitation, and of this again 3.0 at the very least as due to the betterment of the water by filtration. In this there have been reckoned for the infants under one year only 1.8 instead of 2.7, to avoid by any chance putting the figure too high; for cholera 0.8, and for typhoid fever 0.4 per 1,000. Even leaving the cholera out of account, an average improvement by at least 2.2 per 1,000 must be acknowledged. That gives, even with this thoroughly cautious and conservative calculation, for a population of, in round numbers, 340,000 (as the number stood in 1872), an annual number of deaths 748 fewer, and for the present population of 700,000, 1,540 deaths fewer in a

[1] *Die Gesundheitsverhältnisse Hamburgs im neunzehnten Jahrhundert*, den ärztlichen Theilnehmern der 73. Versammlung deutscher Naturforscher und Aerzte gewidmet von dem Medicinal-Collegium, Hamburg (Verlag von Leopold Voss), 1901. Translating freely, we shall apply to this work the English title given above—the term "history" being suggested by Dr. Reincke's preface. As the sections from which we quote bear no signatures, we assume that these sections are editorial and hence to be credited—at least in the main—to Dr. Reincke; many of the statements which we have quoted from his Annual Reports are, in fact, either paraphrased or amplified in the later work. In making this assumption we hope that we do no injustice to any of Dr. Reincke's collaborators.

year, which are to be accredited to the filtration of the drinking water. Undoubtedly, however, the real figures are much higher.

Through the improvement of the water-supply already discussed and the other contemporaneous measures of sanitation, Hamburg has caught up with most of the larger German cities, which hitherto had had more favorable sanitary conditions, and has surpassed many (pp. 310, 312).

2. Infant Mortality, Diarrhea, and Gastro-intestinal Diseases.

The term "Infant Mortality," as we shall use it here, has a special significance corresponding to the German "Saüglingssterblichkeit, d.i. der Sterblichkeit der im ersten Lebensjahre stehenden Personen," i.e., the mortality of infants under one year of age. As 90 per cent of all deaths from diarrhea and gastro-intestinal diseases (Durchfall und Brechdurchfall) in Hamburg in the nineteenth century occurred among infants under one year of age, we combine these heads as above.

It is certainly in consequence of sanitary improvements that, in spite of the growing factory-working population of the city, the *infant mortality has declined so extraordinarily since 1893*. And as a discussion, beyond, on gastro-intestinal diseases will show, *this abrupt and lasting improvement is to be attributed solely to the filtration of the drinking water* (Diese plötzliche und andauernde Verbesserung ist allein der Filtration des Trinkwassers zu danken) (p. 145, italics ours).

Under diarrhea and gastro-intestinal diseases it is noted that the mortality under this title is not statistically complete, since most of the deaths of infants from atrophy and debility (Atrophie und Lebensschwäche) really belong here. The evidence for this is found in their parallelism in seasonal and yearly prevalence.

Manifestly the as yet unidentified germs of these diseases are ingested by infants with their nourishment—never, however, in mother's milk, but only in artificial substitutes, especially cow's milk—which in some way or other has been contaminated with pathogenic germs. . . . . Among the dangerous contaminations of milk, a great part was played in Hamburg, until 1893, by the unpurified Elbe water, which was introduced into all the dwellings by the city water system. . . . . But since the water has been filtered, a gratifying decline in infant mortality has set in [see our Chart 3] (pp. 149, 150).

Since diarrhea and gastro-intestinal diseases attack chiefly infants under one year [who contributed 90 per cent of the deaths from these causes in Hamburg in the nineteenth century], and as no other diseases of infants claim nearly as many victims, it is evident why the century curve for these causes of death shows an extraordinary similarity to the curve of total infant mortality. Divergences occur only in exceptional years when for a time infants died in increased numbers from other diseases, or adults from diarrhea (p. 207).

The data given below concerning the connection of disease with drinking water suggest, moreover, the question whether the rise of the century curve since the '60's does not stand in a definite connection with the extension of the public water-supply of unfiltered Elbe water. The mean curve by months agrees, as would be expected, almost completely with the curve of deaths of infants from gastro-intestinal diseases. . . . . It virtually follows the atmospheric temperature, for continuous hot days usually unmistakably increase the number of cases of sickness. This subject, however, still needs for complete clarification much more thorough investigation than it has yet had. It must not be forgotten that hot days in May and June scarcely increase gastro-intestinal disorders. Furthermore, in midsummer the temperature is not the only decisive factor, for atmospheric humidity, the distribution of storms, and other factors which are of influence either upon the dissemination of pathogenic organisms or upon their multiplication in milk and other foods, also co-operate.

To *one* of the factors, which plays a part in the dissemination of the germs, attention has been drawn in Hamburg by the fact that the mean curve by months shows here a second rise, which, tho small, nevertheless reached in certain years (e.g., in January, 1883, 1890, 1892; in February, 1889; and in December, 1885, 1886, 1888, and 1891) a very considerable height. This rise occurred always in the winter, but cannot possibly be explained by colds, by life in small, badly ventilated rooms, by injury of milk consequent on heating, or by damage done by Christmas feasting; for, so far as we have been able to determine, in other places of similar climate, the winter increase in gastro-intestinal disorders does not, under ordinary circumstances, take place. Moreover, the case is very different in Hamburg in the various years. And above all, it has ceased since the city water has been well filtered (Und vor Allem da sie aufgehört hat, seitdem das Wasser der städtischen Wasserleitung gut filtrirt wird).

The suspicion cast upon the unpurified Elbe water by these facts is strengthened by observations in other places. In this connection what happened in the district of the Stralau waterworks at Berlin[1] is of great interest. When the waterworks delivered badly filtered water, the population of the district supplied from Stralau responded at once with an increased mortality of infants from gastro-intestinal diseases, which occurred moreover exclusively among infants artificially nourished. A similar observation was recorded in the report mentioned (p. 13) regarding the Altona[1] waterworks as early as 1870, and it was further stated that every time disturbances took place in the operation of the filters at the Altona works, the prevalence of gastro-intestinal disorders increased in the city. Finally, it was recounted that after several high tides in November and after an especially high "storm-tide" on the 4th of December, 1892, bringing about a heavy pollution of the Elbe water at the waterworks intake (proved bacteriologically), an increased infant mortality occurred in Hamburg. The bacterial content of the Elbe River water rose at this time from 200–300 per c.c. to 7,500. Such observations led of necessity to the conclusion, that in other years also when similar events took place the polluted city water was to blame. As already stated (p. 50) there is a possibility that filth particles and pathogenic germs emptied into the Elbe with sewage are driven occasionally by the tide upstream, both to the old and the new waterworks intakes. When this happened there undoubtedly followed as a rule a pollution of the public water-supply, until this danger was eliminated by the establishment of sand filtration and the regular stoppage of pumping at high tide. This danger

[1] For fuller accounts of the phenomena at Berlin and Altona see *Jahresb. über die medizinische Statistik des Hamburgischen Staates fur 1892;* translations given above, pp. 494–97.

was not always of the same magnitude, but varied both according to the amount of fresh water coming down, which made difficult or easy the upstream movement of the tide-wave, and also according to the height of the tide-wave coming in from the sea and influenced by wind and weather (pp. 208-9).

Next follows a description of float experiments, by which it was determined how high the tides would have to be, at various stages of the river, to drive water from the outfall of the main trunk sewer upriver to the old and (since 1893) new waterworks intakes, located 6.3 and 8.5 km. upstream, respectively.

Of course the summer months, with their lower river levels, are by far the most dangerous; there appear, however, extraordinary differences in individual years. On the one hand, there are wet summers with proportionately high fresh water stages, and, on the other, dry years in which the level of the river sinks very low in the hot season. There are also mild, rainy winters in which the river remains high, as well as those in which the whole river-basin freezes up and all the precipitation remains lying on the ground as snow, so that the flow of the river becomes very scanty. Hence for our purposes the separate years must be closely studied (p. 210).

Then follow tables which lack of space forbids us to reproduce, showing, for the period of 1877–93, the varying stages of the river and the exceptionally high tides, and the time relations of these two sets of phenomena.

If the data in these tables be now compared with the tabulations of the contemporaneous deaths from gastro-intestinal diseases (p. 220), it appears clear that the dry years, with subnormal river stages in the late summer and fall (1877, 1878, 1883–1887, and 1892) and with numerous unfavorable tides, have been especially characterized by deaths from these diseases; but that in the driest year of all, 1893, the significant rise in deaths which was to be expected did not take place. It must be concluded from this that low river stages and higher prevalence of these diseases are not merely independent parallel consequences of a greater summer heat, for in that case gastro-intestinal disorders would have been worse than ever in 1893; but that since 1893 there has been broken a link in the chain which previously connected summer gastro-intestinal disorders with the low water. And that break has come with the filtration of the drinking water begun in 1893. This is confirmed, moreover, by observations of all subsequent years down to the end of the century, during which the low waters of the autumns of 1895, 1898, and 1900 have had no perceptible influence whatsoever upon gastro-intestinal disorders.

Without doubt, therefore, the summer gastro-intestinal disorders of children have been largely increased by the city water, so long as this was not filtered The process may be pictured as consisting simply in the dissemination of the infective agents in kitchens, on utensils, on bottles, on the hands of mothers and nurses, or in the milk itself, in greater quantities through the water than in other ways. The germs then multiply more or less, according to the cleanliness of the people and the condition of their dwellings and according to the weather, and, in greater or less numbers, are ingested by infants with their food.

The conditions favoring gastro-intestinal disorders in winter are not so clear, altho these also threw suspicion on the water. It is not difficult, in fact, to discover for those winter seasons in which the deaths from gastro-intestinal diseases rose abnormally, storm-tides upon which the blame could be laid; but in our table are found many high tides which apparently had no effect upon these diseases—in particular, all the storm-tides of the autumn equinox. From these a sceptic could derive justification for disputing altogether the connection between polluted water and gastro-intestinal disorders; he could also adduce the fact that before the sewerage of Hamburg and before the introduction of the public water-supply winter increases in gastro-intestinal diseases had appeared, e.g., in 1838 and 1845; and that even after the establishment of filtration, e.g., in March, 1895, there had been observed once more an increase in deaths from these diseases.

On the other hand, we may reply that the winter rises in the '30's and '40's took place in such small numbers that inferences cannot safely be drawn from them. And if any increase of these diseases did in fact take place in those years, that would not prove that the Elbe water was not to blame, for, in fact, before 1848 water was freely taken from the river, which, in former times, may have been badly polluted upstream. . . . .

Neither can the rise of gastro-intestinal diseases in March, 1895, decide the question, if the scant excess of deaths be given due weight, for in those first years of filtration the management of the waterworks still had difficulties, now entirely overcome, to contend with, arising in very cold weather with the cleaning of the filters. It appears, in fact, from reports in my possession on the bacteriological control of the filters in 1895, that in February and March of that year individual filters repeatedly worked imperfectly, so that a few of the infective agents of these diseases may have slipped through. . . . .

On comparing the two tables on page 211 and page 220, it is found that the high stages of the river caused by melting snow [at certain times] did not have the slightest influence on the number of deaths from gastro-intestinal diseases, while in January, 1883, as also in the beginning of the year 1845, the increase of deaths from these diseases coincides with a much swollen river. Before drawing any conclusions from these observations, further experiments must be awaited, and the conditions in other cities be thoroughly compared.

Meanwhile we must be content to have established the fact that the unpurified city water has, until the inception of filtration, contributed heavily to the spread of gastro-intestinal diseases (pp. 210–16). [The italics are ours.]

The decline in infant mortality is of special interest because in this we see a result of the filtration of the drinking water which has exceeded all expectation and has materially enriched our knowledge of the propagation of gastro-intestinal disorders. . . . . If therefore the whole improvement . . . . cannot be ascribed to these diseases and hence to the results of better water, certainly the overwhelming majority of cases also belongs in this category (p. 310).

We quote also the following with reference to the relation between gastro-intestinal disorders and typhoid fever:

Formerly, disturbances in the operation of the filters at the Altona waterworks repeatedly appeared, especially in very cold weather, which made the proper cleaning of the filters difficult or impossible. At such times unfiltered or insufficiently filtered

water entered the distribution system, whereupon the health of the people would react at once with the appearance of cases of gastro-intestinal disorder, a sure sign to the Altona physicians that two to three weeks later typhoid cases would follow (p. 234).

Examples of such outbreaks are given, characterized as sudden in onset, restricted to the Altona district, falling in seasons otherwise unfavorable to typhoid propagation, and as having been completely absent since the improvement of filtration in Altona.

A similar local and explosive typhoid outbreak occurred in Hamburg in 1893 ("Jahresbericht für 1893," p. 55). On a wharf on the south bank of the Elbe, where the laborers were provided, for drinking purposes, with Elbe water purified by a filter of special construction . . . . 100 laborers suddenly fell sick with gastro-intestinal disorder, and 19 of them developed typhoid. Closer investigation revealed the fact that shortly before the outbreak of the sickness the filter had been "cleaned" and that its efficiency had for a while been very much reduced (p. 235).

. . . . Instances from the winter season are much more characteristic, for then the influences of the summer heat have quite ceased. A few are summarized in the table below [Table 3].

[TABLE 3.]

DEATHS FROM GASTRO-INTESTINAL DISEASES AND CASES OF TYPHOID FEVER, BY WEEKS.

	WEEK NUMBERS [FROM BEGINNING OF EACH YEAR].																				
	41	42	43	44	45	46	47	48	49	50	51	52	1	2	3	4	5	6	7	8	9	10
1888–89																						
Gastro-intestinal diseases	28	**57**	31	21	15	10	17	**43**	**46**	36	28	24	17	11	15	10	43	54	**83**	60	21	..
Typhoid fever	42	32	46	**83**	46	54	32	33	46	**92**	**75**	48	41	50	40	30	45	41	55	80	**112**	57
1890																						
Gastro-intestinal diseases	..	..	..	..	..	..	..	..	..	..	..	..	17	24	17	42	**64**	**67**	29	17	12	8
Typhoid fever	..	..	..	..	..	..	..	..	..	..	..	..	34	35	34	28	35	35	**48**	44	43	24

From this it cannot be doubted, keeping in mind the foregoing instances and similar cases elsewhere (e.g., at the Tegel waterworks in Berlin, January to March, 1889 [account already quoted from "Jahresbericht für 1892"]), that typhoid fever and gastro-intestinal diseases are to be attributed to a common vehicle which can only be found in the public water-supply (p. 238).

3. Tuberculosis.

Especially surprising was the improvement [as to phthisis] in the period from the fourth to the sixth decade, when the sewerage and water-supply systems just completed must have been of very strong influence for general cleanliness (p. 285).

In tuberculosis the decline in mortality began long before 1872; but this progressive improvement has gone on at a distinctly brisker rate since the middle of the eighth decade, due no doubt to the fact that after the discovery of the tubercle bacillus the measures already in operation were more systematically applied. At the same time

it is worthy of notice that it is maintained from the surgical side that since the filtration of the water the number of cases of bone and joint tuberculosis has diminished extraordinarily. Further observations should tell whether a causal connection really exists here. At any rate such a possibility cannot be excluded, since of course the sputa and bowel discharges of all the tuberculous reach the Elbe through the sewers, and tubercle bacilli just as well as typhoid bacilli could have been carried thence in the water to the people (p. 309).

4. Other Infectious Diseases.

In reference to "Inflammatory Diseases of the Respiratory Organs" ("Entzündliche Erkrankungen der Atmungsorgane") (not including phthisis):

Unfortunately such a variety of diseases must be included under this title that only a very general orientation is possible (p. 101).

The decline in these diseases since 1893 is very striking. Prior to that time the annual death-rate from these causes was 3 to 4 per thousand inhabitants, but now the mortality has dropped to 2 to 3 per thousand. What has worked this revolution is difficult to determine, as the picture of events [see diagram] is much confused by the large influenza epidemics of 1891, 1895, and 1900 (p. 103).

The conditions with respect to diseases of the respiratory organs (exclusive of tuberculosis) are even more obscure [than for tuberculosis]. Still the striking improvement [shown by comparative rates for the two periods 1872–96 and 1896–1900] in this group, which includes very diverse forms of disease, must be accredited more to general sanitary improvements than to the filtration of the water (p. 310).

It is interesting to note that of all deaths from inflammatory diseases of the respiratory organs in the period 1872–1900, about 25 per cent occurred among infants under one year of age (pp. 133, 160).

In the quotations here given we believe that we have cited all of special interest or importance in reference to the effect of a purified water-supply upon the various infectious diseases in Hamburg. And we desire, on leaving this portion of the subject, to express our high appreciation of the thoroughness of the work of Dr. Reincke and his associates, which has thrown so much light upon this important subject.

VI. MR. MILLS'S FURTHER STUDIES ON THE WATER-SUPPLY AND TOTAL DEATH-RATE, ETC., OF LAWRENCE, MASSACHUSETTS, AND HIS ADDRESS TO THE CITY GOVERNMENT OF LAWRENCE, 1893–1902.

Ever since 1893 Mr. Mills had been making further observations and studies, some of which he had reported to his colleagues on the State Board of Health of Massachusetts as early as 1896. Several

years later (July 3, 1902) in the course of an address before the city government of Lawrence, he read a statement of his results, from which we may quote the following striking paragraphs, published the next day in the Lawrence newspapers:

The deaths from typhoid fever dropped from an average of 52 per year for the six years previous to the use of the filtered water to an average of 12 per year in the past six years. But this is only a small part of what has been accomplished. The deaths from all diseases in the city have, since the filter was built, averaged 19 annually per thousand of inhabitants, while in the six years previous to the building of the filter the deaths from all diseases averaged 24 per 1,000 annually. That is, in the years since you began to use filtered water there has been an average of 300 less deaths per year than there would have been if the death-rate had continued as before. . . .

When the United States Census Bureau of 1900 found that the deaths in Lawrence were, when they considered the increase in population, equivalent to more than 300 less deaths than in 1890, they wrote to your City Board of Health asking the cause, and were informed that the principal cause that they could indicate was the introduction of filtered water for the use of the city in 1893. They had reason for their conclusion when their records showed the deaths in 1892 to be 1,246 and the deaths of 1894 to be 925; a difference of 321 deaths from the year before to the year after the filter was completed.

Since you began to drink filtered water the records show that there have been as many as 2,500 less deaths in the city than there would have been if the average death-rate of the previous six years had continued to this time; and this has been one of the sources of increase in population. . . .

The decrease in deaths has not been limited to deaths caused by diseases which are known to be conveyed by water, nor to infectious diseases, but includes so broad a range of diseases that we must conclude that the general health of the people has been improved and their ability to resist and overcome disease has increased.

VII. DISCUSSION BY MR. ALLEN HAZEN, C.E., OF THE WATER-SUPPLY OF LAWRENCE IN RELATION TO THE TOTAL DEATH-RATE, 1901.

In the published discussion of a paper[1] presented by Messrs. Morris Knowles and C. G. Hyde at a meeting of the American Society of Civil Engineers, June 5, 1901, Mr. Hazen made the following statement:

While the most striking reduction in the death-rate following the use of filtered water was in typhoid fever, the general death-rate showed a very considerable reduction. Table No. 26 shows the death-rate per thousand living for the seven years that the filter has been in operation, together with the corresponding rates for three preceding periods of seven years each.

The reduction in the general death-rate in the last seven years, as compared with the seven preceding years, is several times as large as the reduction in the typhoid

[1] The Lawrence, Mass., City Filter: A History of Its Installation and Maintenance, *Trans. Am. Soc. C.E.*, 46, p. 316.

fever death-rate, and perhaps indicates the effect of the quality of th diseases. The question as to how far this is due to the water-supply improved general sanitary conditions, may be properly raised; but reduction at the time of the introduction of the filter, and the steadiness rate has continued on a lower level since, seem to indicate that the w something to do with the reduction, and that the coincidence was not a

VIII. A GRAPHICAL DEMONSTRATION OF THE LAWRENCE PUBLISHED BY MR. MILLS, 1904.

In April, 1904, Mr. Mills showed to one of the autho who was about to give in Lawrence a public address supply of that city, the diagram herewith reproduced of recent years by the authors) as Chart 1, and kin him to make from it a lantern slide which he then since used from time to time in his lectures. This dia was afterward published with slight additions in the An of the Water Board of Lawrence for 1906, illustrates striking way the Mills-Reincke phenomenon to which we have now repeatedly referred.

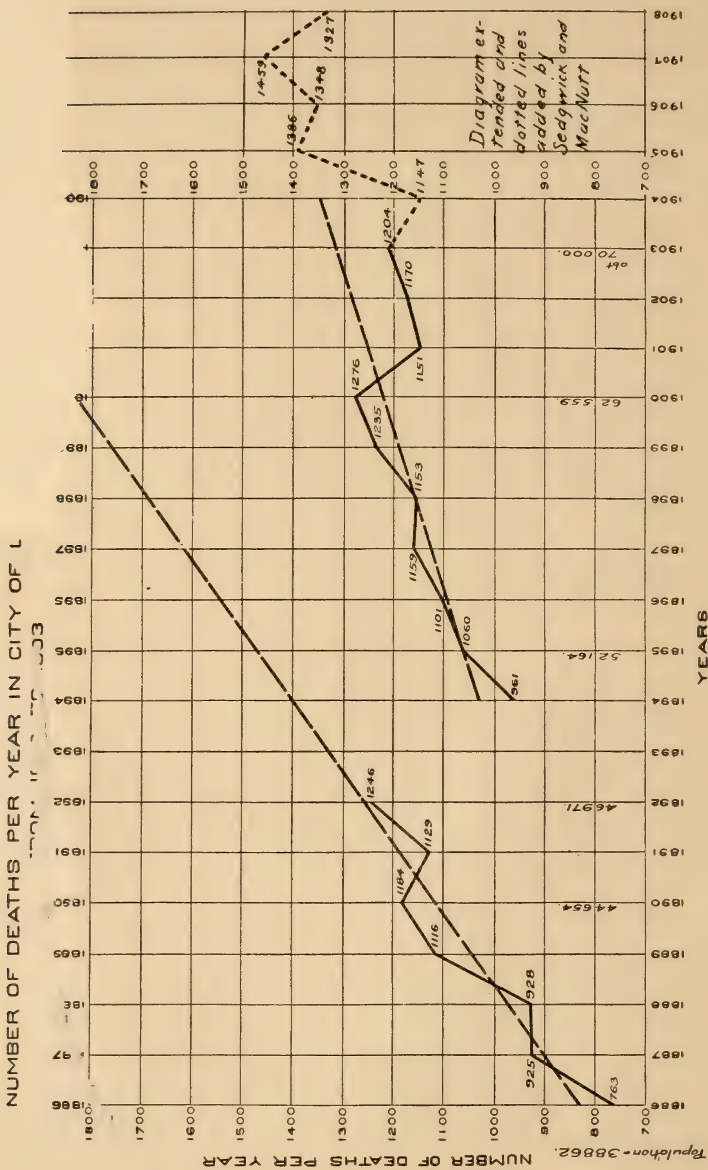
IX. FORMULATION OF HAZEN'S THEOREM, 1904.

At the International Engineering Congress held at St. Louis in October of the same year, Mr. Allen Hazen took an important step in advance, and formulated a numerical expression for the comparative effect of water purification upon typhoid fever mortality and total mortality. He had already approximated such an expression in his statement made in 1901 and quoted above that—

> the reduction in the general death-rate . . . . is several times as large as the reduction in the typhoid fever death-rate.

On account of its importance as the first attempt, so far as we are aware, to reduce to a quantitative statement the Mills-Reincke phenomenon, we shall quote in detail that part of Mr. Hazen's study which concluded with the formulation as above mentioned of a numerical expression of the relation of total mortality to typhoid fever mortality.

> The general death-rate, as might be expected, is also reduced, and it is reduced to a greater extent than can be accounted for in the reduction of the typhoid fever rate. The percentage of reduction in the general death-rate is less than it is with typhoid


NUMBER OF DEATHS PER YEAR IN CITY OF L
NUMBER OF DEATHS PER YEAR
YEARS
Population - 38862.
Diagram extended and dotted lines added by Sedgwick and MacNutt
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
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1905
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1300
1200
1100
1000
900
800
700
763
925
928
1116
1184
1129
1246
961
1060
1101
1159
1153
1235
1276
1151
1170
1204
1147
1386
1348
1459
1327
44 654.
46 971.
52 164.
62 559.
70 000.

Chart 1.

fever and it is therefore more difficult to follow the relation, and the reduction attributable to the change in water can be ascertained with less certainty.

Tables 5 and 6 show the general death-rates and the death-rates from typhoid fever in a number of cities, before and after radical improvements in their water-supplies. In four cases, two in Europe and two in the United States, the changes were made by the installation of sand filters. In the other cases the changes were from polluted river supplies to upland waters from unpolluted sources, or to ground-water. The year of change (when both the old and new supplies were in use) is omitted in each case. At Lowell, Mass., where the change was more gradual, two years are omitted, and at Hamburg, the cholera year, 1892, which immediately preceded the year in which the filters were put in service, is also omitted from the comparison.

TABLE 5.

DEATHS FROM TYPHOID FEVER PER 100,000 PER ANNUM.

Place	Date of Change	Five Years before Change	Five Years after Change	Percentage of Reduction
Zurich, Switzerland .... Filtration	1885	76	10	87
Hamburg, Germany .... Filtration	1892–93	47	7	85
Lawrence, Mass .... Filtration	1893	121	26	79
Albany, N.Y. .... Filtration	1899	104	28*	73
Lowell, Mass., river water to ground-water	1895–96	97	21	78
Newark, N.J., river water to upland water	1892	70	16	77
Jersey City, N.J., river water to upland water ..	1896	77	24	69
Averages		85	19	78

* Four years.

The reductions in death-rates with the installation of filters have been quite as great as in those cities where the new sources of supply were from unpolluted sources. The average reduction in the typhoid rate was 66 per 100,000. The reduction in the general death-rate was 4.7 per 1,000 or 470 per 100,000.

TABLE 6.

DEATHS FROM ALL CAUSES PER 1,000 PER ANNUM.

Place	Date of Change	Five Years before Change	Five Years after Change	Percentage of Reduction
Hamburg, Germany .... Filtration	1892–93	24.0	17.7	26
Lawrence, Mass .... Filtration	1893	24.4	20.0	18
Albany, N.Y. .... Filtration	1899	22.3	18.4*	17
Newark, N.J., river water to upland water	1892	25.1	22.1	12
Jersey City, N.J., river water to upland water ..	1896	25.4	19.3	24
Lowell, Mass., river water to ground-water	1895–96	25.1	20.5	18
Averages		24.4	19.7	19

* Four years.

While the reduction in the typhoid rate is 66 per 100,000, that in the general death-rate is 4.7 per 1,000 or 470 per 100,000, or seven times as much. Where there is one less death from typhoid fever there are six less from other causes.

The writer believes that the whole of the reduction in the typhoid rate should be attributed to the change in water-supply, because cities similarly situated, which have

not improved their supplies, have experienced no permanent reduction in their typhoid fever rates. With the general death-rate the case is different. Improved general sanitary conditions have reduced the death-rates in recent years, and the normal reduction in a period of six years, which represents the average elapsed time between the first and second series of results, would account for a part of the reduction in the general death-rate.

The average reduction in the general death-rate between 1890 and 1900 in eighteen cities having from 50,000 to 300,000 inhabitants, in New England, New York, and New Jersey, which made no radical change in their water-supplies, was 2.28 per 1,000. This is computed from the Report on Vital Statistics in the United States Census of 1900. Assuming a uniform decrease in rate in the interval, the average, or what we may call the normal, reduction, in 6 years would have been 0.6 of this, or 1.37 per 1,000. In comparison with this, in five cities where the water was radically improved, the reduction in the same period was 4.4 per 1,000. The results may be tabulated as follows:

	Death-Rate per 100,000 Living
Reduction in total death-rate in five cities with the introduction of a pure water-supply	440
Normal reduction due to general improved sanitary conditions, computed from average of cities similarly situated but with no radical change in water-supply	137
Difference, being decrease in death-rate attributable to change in water-supply	303
Of this, the reduction in deaths from typhoid fever was	71
Leaving deaths from other causes attributable to change in water-supply	232

This computation indicates that *where one death from typhoid fever has been avoided by the use of better water, a certain number of deaths, probably two or three, from other causes have been avoided.* [Italics our own.] This seems the clear and logical conclusion from the statistics. It is not easy to explain how the water is connected with the deaths other than those from typhoid fever. It may be that a good water-supply, used freely and with confidence, results in a better general tone in the systems of the population, and so indirectly to a lower death-rate, and that a part of the reduction is represented by diseases having no recognized connection with the quality of the water-supply ("Trans. Am. Soc. C.E.," 54D pp. 151–53).

The quantitative relation thus arrived at by Mr. Hazen and italicized above, seems to us to deserve, because of its far-reaching importance, some special designation. We have therefore ventured to apply to it in our preliminary paper[1] the term *Hazen's Theorem.*

Mr. Hazen further added, in the discussion on this paper:

The writer agrees with Messrs. [E. O.] Jordan and G. W. Fuller that it is most important that further study be made of the effect of water-supply upon other diseases than typhoid fever. The statistics certainly indicate a much wider influence of water-supply upon public health than has been generally admitted; and it would seem as if studies might be made to ascertain the facts more definitely (*ibid.*, p. 251).

[1] *Science*, August 14, 1908.

X. OTHER DATA AND DISCUSSIONS BEARING ON THE GENERAL SUBJECT.

We have not been able to find later than the formulation in 1904 of Hazen's theorem any definite advance in the analysis of the underlying Mills-Reincke phenomenon or any attempt at a general proving of the theorem itself. It enunciation has, however, caused occasional discussion and called forth some confirmatory data from various quarters. Without attempting to quote all such data and discussions we shall summarize the more important.

Professor Edwin O. Jordan, in the discussion on Mr. Hazen's St. Louis paper, made the following remarks:

> Mr. Hazen's computation which indicates that, in addition to the reduction in deaths from typhoid fever, there occurs a similar reduction in deaths from other causes, and that this also is attributable to a change in the water-supply, is certainly very striking. There is one suggestion which the writer would like to offer concerning a possible explanation of this circumstance.
>
> It is well known that the obscure character and variable symptoms of certain cases of typhoid fever lead not uncommonly to a mistaken diagnosis. There is no doubt that in every considerable body of vital statistics some cases of genuine typhoid fever are reported under other names. It need hardly be said that this is particularly true as regards the deaths reported under the captions "typho-malarial fever," "malarial fever," and similar designations. In the Northern United States, the majority of the deaths reported under these headings are deaths from typhoid fever. The effect of filtration well illustrates this point. In the city of Albany, N.Y., there were 37 deaths reported under the heading "malarial diseases" in the four years, 1891–94, and 23 deaths in the four years, 1895–98, while for the four years following filtration (1900–1903), there were but two deaths under the same head.
>
> It must be remembered also that a marked improvement in methods of diagnosis of typhoid fever has taken place during the last decade. One result of this has been that deaths formerly reported as occurring from other causes are now correctly reported as due to typhoid fever. This transfer to the column of typhoid deaths naturally diminishes the deaths reported from general causes and must, at least, partly explain the relation pointed out by Mr. Hazen. It may also serve to explain why the apparent reduction in deaths from typhoid fever is sometimes less than would be reasonably anticipated. The suggestive facts brought out in the paper indicate that other items under the reported deaths in official health reports might repay examination ("Trans. Am. Soc. C.E.," 54D, pp. 206, 207).

Three years later the phenomenon observed at Lawrence by Mr. Mills was referred to in a paper[1] by Mr. H. W. Clark, who called attention to a similar decline following the introduction of filtration at Albany, N.Y.

[1] *Monthly Bull. Mass. State Board of Health*, Sept., 1907, p. 242.

On November 6, 1907, Mr. Edward E. Wall, in a paper[1] presented before the American Society of Civil Engineers, wrote as follows:

Allen Hazen, M. Am. Soc. C.E., estimates that where one death from typhoid has been avoided through an improved water-supply probably two or three deaths from other diseases have been avoided. The following record seems to indicate a higher proportion of decrease than Mr. Hazen's estimate would give:

ANNUAL MORTALITY RECORD, ST. LOUIS, MO., 1900–1906, FROM DISEASE ALONE.
[Chemical precipitation introduced in 1904.]

1900	9,217	1904	10,695
1901	9,916	1905	9,545
1902	9,654	1906	9,214
1903	10,320		

Mr. Mills's observation of the phenomenon at Lawrence was again referred to by Mr. Stephen DeM. Gage in the discussion on a paper on filter operations at Lawrence, Mass., presented by Messrs. M. Knowles, M. F. Collins, and A. D. Marble before the New England Water Works Association, February 12, 1908:

In addition a very material reduction has occurred in the total death-rate of the city, a reduction so different in character from that gradual decrease due to increased appreciation of municipal sanitation which has taken place throughout the State of Massachusetts and following so closely the introduction of filtered water that it can only be explained by attributing it to the improvement in water-supply ("Jour. N.E. Water Works Assn.," 22, p. 232).

Mr. Gage accompanied his statements with a diagram showing the decline in the typhoid fever death-rate and in the total death-rate at Lawrence for the period 1880–1905.

Finally, as the only other reference we have been able to discover, we may record the observation based on statistics, by Mr. E. L. Grimes, City Engineer of Troy, N.Y., and reported at the same meeting, that following the extension and sanitary improvement of the water-supply of that city there occurred a distinct decrease, not only in the deaths from typhoid fever, but also in the deaths from cholera infantum, diarrhea, diseases of the digestive organs, and of children under five years of age.[2]

XI. A RECAPITULATION OF THE LITERATURE, AND A BRIEF OUTLINE OF THE WORK OF THE AUTHORS.

A review of the foregoing sections leads irresistibly to the conclusion that Messrs. Mills, Reincke, and Hazen, in their observations

[1] "Water Purification at St. Louis, Mo.," *Trans. Am. Soc. C.E.*, 60, p. 182.

[2] *Jour. N.E. Water Works Assn.*, 22, p. 183.

and studies already described, have brought to light phenomena of the very first importance in sanitary science. There appears to be no escape from the conclusion that the purification of polluted public water-supplies reduces the general death-rate much more than it would be reduced by the saving of deaths from the commonly recognized water-borne diseases, typhoid fever and Asiatic cholera, alone. The question naturally arises, to what extent is the general death-rate reduced? And this simple question Hazen's theorem undertakes to answer. Another question which naturally arises is, in what particular diseases does this reduction take place? And this question we shall ourselves undertake to answer.

As yet very few students of public health science are aware of the extent of the studies of Reincke and Mills; and no one, so far as we know, has hitherto undertaken any thorough confirmation of their results. We have therefore ventured to make a study of this kind, first critically examining all the evidence in our possession relating to the Mills-Reincke phenomenon and Hazen's theorem; and then, having found the latter sound and conservative, passing on to the study of fresh examples, to test its scope and range.

Following the historical development of the subject we have naturally begun with the data afforded by the city of Hamburg.

XII. A GRAPHICAL DEMONSTRATION AND DISCUSSION, BY THE AUTHORS, OF THE HAMBURG PHENOMENA.

As a detailed demonstration of the mortality phenomena before and after the introduction of filtration at Hamburg we have taken statistics from the "Sanitary History of Hamburg in the Nineteenth Century" and plotted from them in Chart 2 the curves for certain important causes of death. On the vertical lines, corresponding to the middle of each year, are plotted the average annual death-rates. The period of unfiltered water is indicated by the heavy black bars beginning on the left and crossing more than one-half the diagram. The period of filtered water-supply is indicated by the clear spaces following these heavy black bars. The scale employed, it must be observed, is the same for all the curves except the two lowest, thus facilitating comparisons. In order to save space, the unused portion of the scale below each curve except the uppermost has been cut off.

Hamburg is well known in the annals of sanitary science on account of its great Asiatic cholera epidemic of 1892. A slow sand filter was at that time in process of construction, and the epidemic of cholera caused a precipitate activity in construction which resulted in the rapid completion of the filter and the beginning of its operation in May, 1893. A brief description of the water-supplies of Hamburg and the neighboring city of Altona is given by Dr. Reincke in his Annual Report for 1892 and translated by Mr. Hazen in an appendix to his "Filtration of Public Water-Supplies." A fuller account is given in the "Sanitary History of Hamburg in the Nineteenth Century." We need only recall that previous to the introduction of filtration in Hamburg the public water-supply was taken from the (tidal) Elbe River at a point about four miles above the outfalls of the main trunk sewers of Hamburg and Altona. In 1893 the intake was moved about a mile farther upstream, and while the river at these points was probably not ordinarily heavily polluted, Dr. Reincke brings conclusive evidence (quoted by us, pp. 495–97, 500–503) to show that at certain times of high tide and low river the sewage of Hamburg and Altona was driven upstream and came at such times directly to both intake points. By filtration the effects of the pollution from this source have been eliminated. As the diagrams of death-rates in Chart 2 speak for themselves, we need comment on them only briefly.

From the first curve it will be seen that the typhoid fever death-rate fell from an average of about 30 in the five years prior to 1893 to a rate after filtration of less than 10 per 100,000.

In inflammatory diseases of the respiratory organs there was an extraordinary drop, and the rates from this cause of death have continued on a much lower level since 1893. The fall in the mortality from these diseases is so striking that we have continued the computation of the rates for a few years beyond the last date shown in the plate, and have found that the low level assumed after introduction of filtration was steadily maintained. The following rates are computed from data from the Imperial Health Office Bulletin, "Sterbefälle im Deutschen Reiche"; for 1901, 228; 1902, 215; 1903, 196; 1904, 188; and may readily be added by the reader if he so desires. (To avoid misunderstanding of the method of plotting

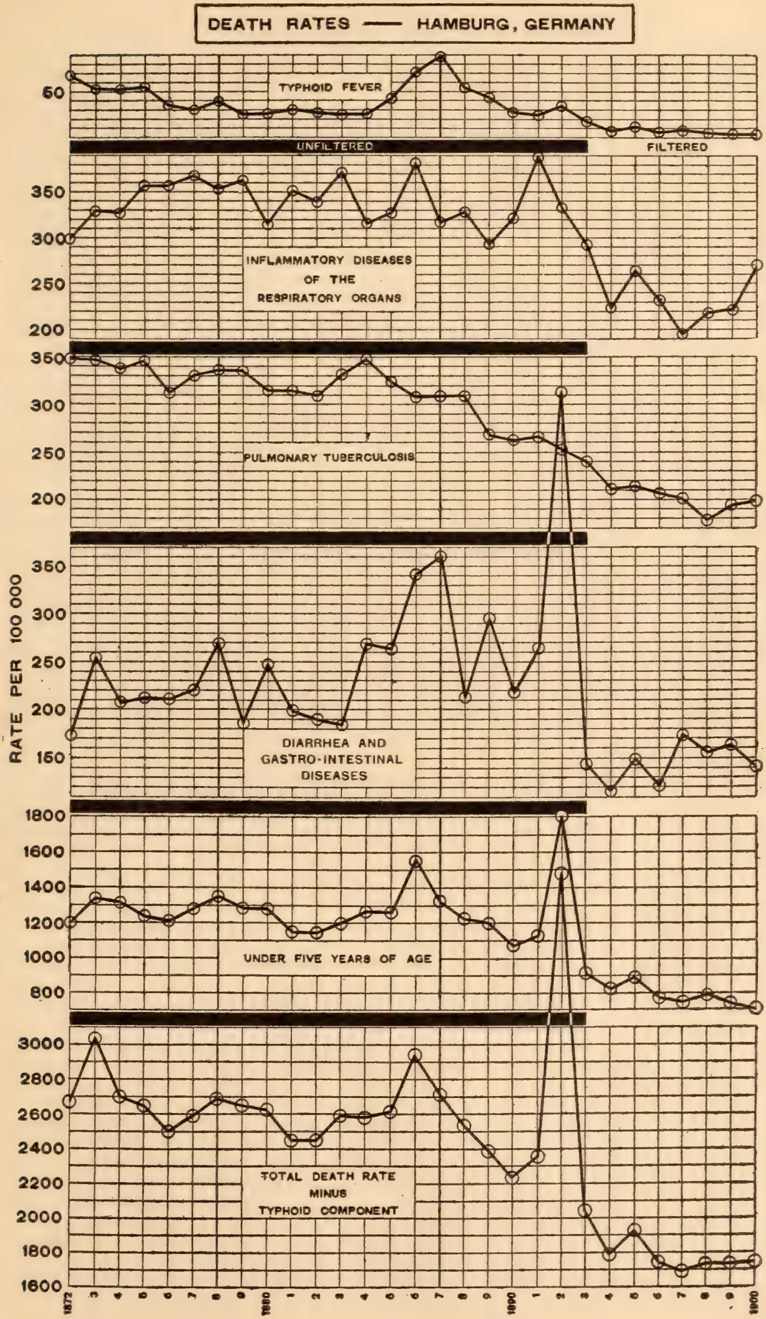
DEATH RATES — HAMBURG, GERMANY
TYPHOID FEVER
UNFILTERED
FILTERED
INFLAMMATORY DISEASES OF THE RESPIRATORY ORGANS
PULMONARY TUBERCULOSIS
DIARRHEA AND GASTRO-INTESTINAL DISEASES
UNDER FIVE YEARS OF AGE
TOTAL DEATH RATE MINUS TYPHOID COMPONENT
RATE PER 100 000
50
350
300
250
200
350
300
250
200
350
300
250
200
150
1800
1600
1400
1200
1000
800
3000
2800
2600
2400
2200
2000
1800
1600
1872
1880
1890
1900

Chart 2.

we must again caution the reader that the unused scale below this, as below subsequent curves, has been cut off.)

Pulmonary tuberculosis, which had been gradually declining for ten years, showed a somewhat greater rate of decline for the year following the introduction of filtration. Whether the purification of the water-supply played any considerable part in this we are unable, from the Hamburg data alone, to determine, but our studies in later sections of this paper on Lawrence and Lowell, Massachusetts, give good reason for supposing that at Hamburg also the purification of the water-supply had a certain effect in lowering the mortality from phthisis.

The title "diarrhea and gastro-intestinal diseases," as used in Chart 2, corresponds to the German *Durchfall und Brechdurchfall.*[1] The curve for this cause of death shows very strikingly the influence of the change to a purified water-supply. From an average rate immediately before filtration of about 300 it dropped suddenly by a full half, at which level it has since continued to 1900. We are forcibly reminded of Dr. Reincke's remark, already quoted, that "in no other diseases did the favorable influence of the better water-supply introduced in 1893 stand out so clearly as in these."

The curve for the death-rate of children under five years of age shows a similarity to that for diarrhea and gastro-intestinal diseases, including as it does a large number of deaths from the latter. Thus the drop in 1894 and the continued lower level of subsequent years is seen in a degree which, when the difference of the scales is taken into account, is as striking here as in the curve just above. A considerable share of this mortality is also contributed by inflammatory diseases of the respiratory organs, and a certain similarity between the two curves may readily be traced. We again call attention to the fact that the scale in this diagram, like all the others in the chart except the topmost, is not extended fully to the true base line.

Finally, the diagram for total death-rate minus typhoid component illustrates strikingly that drop following the introduction of filtration which we have named the Mills-Reincke phenomenon, as observed at Hamburg by Reincke. It is at once seen how sudden, how great, and how permanent was the reduction in this death-rate.

[1] See note, p. 494.

The rate had reached a miminum in 1900, and was on its way up again when the influence of filtration came into play. How great numerically the reduction actually was, and how far it is to be attributed to the purification of the water-supply, is discussed below.

In order to carry the graphical demonstration still farther, we have plotted in Chart 3 the death-rates for age-groups, which rates are reckoned more accurately—on the population in each age-group—than on the total population. The Mills-Reincke phenomenon is here seen to affect all age-groups—that under one year, however, most notably. This is evidently to be accounted for very largely by the relation of water-supply to diarrheal and gastro-intestinal diseases, as explained by Dr. Reincke (secs. iv and v), 90 per cent of these diseases having occurred among infants under one year of age and constituting about one-quarter of the whole mortality of the latter.

Thus from Charts 2 and 3 it is seen that all of Dr. Reincke's statements on the decline in total death-rate, in infant mortality, and in the other diseases mentioned by him are fully substantiated. More than that, the data afford a basis for going somewhat beyond his statements, which, as we shall show, were characterized by much caution.

It may be objected that there were, possibly, at the time under consideration, declines in the total death-rates of German cities in general; in order, therefore, to show the extraordinary character of the drop at Hamburg we present the table on p. 519 for the ten largest cities of Germany (according to the Census of 1890), showing their average total death-rates for two periods of three years each, before and after the cholera years and introduction of filtration at Hamburg, the corresponding typhoid fever rates also being given.

It is at once evident how remarkable the reductions were at Hamburg, both in typhoid fever and in the total death-rate. Taking the typhoid fever rate as an index of quality of water-supply, Hamburg is shown to have been much worse off than any other city during the first period. In the second period all show declines, but that in Hamburg stands far above them all, having been six times as great as the average

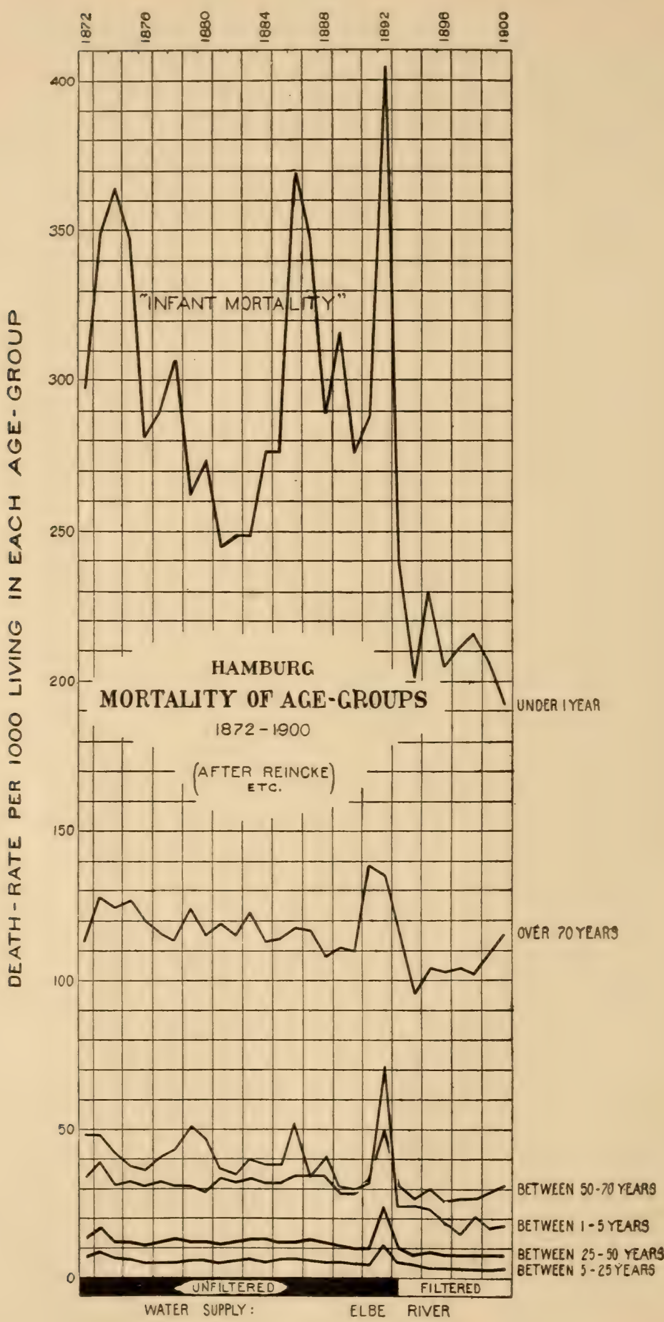
1872
1876
1880
1884
1888
1892
1896
1900
400
350
300
250
200
150
100
50
0
DEATH-RATE PER 1000 LIVING IN EACH AGE-GROUP
"INFANT MORTALITY"
HAMBURG
MORTALITY OF AGE-GROUPS
1872-1900
(AFTER REINCKE ETC.)
UNDER I YEAR
OVER 70 YEARS
BETWEEN 50-70 YEARS
BETWEEN 1-5 YEARS
BETWEEN 25-50 YEARS
BETWEEN 5-25 YEARS
UNFILTERED
FILTERED
WATER SUPPLY: ELBE RIVER

CHART 3.

for the other nine cities. In total death-rate the drop at Hamburg is much greater than that of any other city, and over four times as great as that of the other nine, and this over and above the results of sanitary improvements going on in the other cities.

TABLE 4.

TEN LARGEST GERMAN CITIES	TYPHOID FEVER DEATH-RATES PER 100,000			TOTAL DEATH-RATES PER 1,000		
	1889–91 incl.	1894–96 incl.	Decrease	1889–91 incl.	1894–96 incl.	Decrease
1. Berlin	13	5	8	21.8	20.0	1.8
2. **Hamburg**	**31**	**7**	**24**	**23.1**	**19.2**	**3.9**
3. Munich	8	3	5	27.6	25.2	2.4
4. Breslau	12	8	4	28.8	27.3	1.5
5. Leipzig	13	9	4	20.0	21.7	−1.7
6. Cologne	13	7	6	25.8	24.1	1.7
7. Dresden	8	6	2	20.8	21.0	−0.2
8. Magdeburg	20	9	11	24.6	23.7	0.9
9. Frankfort-on-the Main	8	5	3	18.9	17.6	1.3
10. Hanover	8	7	1	19.6	19.3	0.3
Nine cities exclusive of Hamburg (average)	**11**	**7**	**4**	**23.1**	**22.2**	**0.9**

Typhoid fever death-rates, with exception of Magdeburg, from G. C. Whipple's "Typhoid Fever," App. XVI; other death-rates computed from "Statistisches Handbuch deutscher Städte." The Hamburg figures refer to the *Stadt* exclusive of suburbs, and hence do not quite agree with the rates given by Dr. Reincke for the whole Hamburg district (*Staat*).

Several questions naturally arise at this point. How great was the reduction at Hamburg, not only in the total death-rate, but also in the specific diseases? And what causes contributed most to the total reduction? In answer to these questions we submit in the table on p. 520 a statistical synopsis which we have made of the Hamburg phenomena. The cholera year (1892) and year of introduction of filtration (1893) are omitted, and the means of five years before and five years after are computed, so as to be comparable with our studies of Lawrence and Lowell in sec. xvi.

Of the total reduction, i.e., in the general death-rate, we find that the chief components for specific causes of death are as follows: 19 per cent contributed by diarrhea and gastro-intestinal diseases; 15 per cent by inflammatory diseases of the respiratory organs; 12 per cent by phthisis; 8 per cent by diphtheria; 6 per cent by typhoid fever; and 40 per cent by other causes of death. Viewing the total reduction from the standpoint of age of decedents, we find that 38 per cent falls in the infant mortality under one year of age (as reckoned on the total population).

For every death less from typhoid fever after filtration there were 15.8 deaths less from other causes, but as this figure is merely crude—i.e., has not been corrected for the decrease in mortality that would have taken place independently of water-supply purification—it cannot properly be substituted as the numerical member of Hazen's theorem (see p. 510). It is, however, so much greater than the figure suggested by Mr. Hazen that we are led to venture a partial explanation in the fact that there was not as much typhoid fever to begin with at Hamburg as at Lawrence and Lowell, whether the periods before or after filtration be compared; so that typhoid fever played a smaller part, in proportion, at Hamburg, than in the two American cities upon which, among others, Mr. Hazen's estimate was based.

TABLE 5.

DEATH-RATES, GENERAL AND SPECIFIC, IN HAMBURG BEFORE AND AFTER ADOPTION OF FILTRATION. Computed from the "Sanitary History of Hambuig in the Nineteenth Century."

Cause of Death	Mean Death-Rate 1887–91 Incl.*	Mean Death-Rate 1894–98 Incl.*	Percentage Decrease
All causes	2444	1,774	27
Asiatic cholera	No deaths	except in 1892 and	1893
Typhoid fever	47	7	85
†Diarrhea and gastro-intestinal diseases	270	143	47
†Infant mortality (under 1 year)	304	213	30
Inflammatory diseases of the respiratory organs	329	226	31
Phthisis	283	202	29

* Rates are per 100,000 of population except in the case of infant mortality, for which they are reckoned per 1,000 living under one year of age.

† These two titles overlap; one-quarter of the "infant mortality" is due to "diarrhea," and nine-tenths of the deaths from the latter fall under "infant mortality."

To sum up the Hamburg phenomena, we return again to the striking demonstration of the Mills-Reincke phenomenon as observed there by Dr. Reincke. This consisted in a sudden and permanent drop in the total death-rate minus typhoid component in 1893, an effect observable even in the rate for that year. The whole trend since 1872 of all the curves which we have exhibited, with the exception of that for pulmonary tuberculosis, was changed, each one pitching rapidly and immediately downward and assuming a distinctly lower level thereafter. We refer again to Dr. Reincke's comments on these phenomena as summarized in the preceding pages. We may here anticipate, and say that after our studies on other cities set forth in the following sections, we shall be forced to conclude that—except in respect to

diarrhea and gastro-intestinal diseases and the recognized water-borne diseases, typhoid fever and Asiatic cholera—Dr. Reincke attributed to the purification of the public water-supply much less than its true share in the remarkable reduction in the total and specific death-rates of Hamburg.

XIII. GRAPHICAL DEMONSTRATION AND DISCUSSION OF THE LAWRENCE PHENOMENA

We have made a careful study of the vital statistics of the city of Lawrence, where the Mills-Reincke phenomenon was first observed by Mr. Mills, following the introduction of filtration in 1893. In addition to Lawrence we have taken Lowell, Mass., where the public water-supply was also purified about the same time by a different method, i.e., substitution of unpolluted ground-water for polluted river water. Finally, we have taken the city of Manchester, N.H., as being in every important respect similar (as we shall show) to the two cities just mentioned and therefore an excellent norm for comparison. For all of these cities accurate vital statistics are fortunately obtainable. We have based our computations upon substantial foundations, viz., the populations given by the U.S. Census for 1880, 1890, and 1900, and the Massachusetts State Census for 1885, 1895, and 1905, computing the populations for the intermediate years by the "arithmetical" method of the U.S. Census.[1] The possibility of important errors arising from this method of computing populations for intercensal years will be discussed in section xviii.

The cities of Lawrence, Lowell, and Manchester, all manufacturing cities devoted chiefly to textile industries, are situated in the valley of the Merrimac River, a large, swift stream draining a considerable portion of southern New Hampshire and northern Massachusetts. The first two cities are especially well known through the studies on typhoid fever made there by the Massachusetts State Board of Health, and published in the Report of the Board for 1892. The three cities are so similar in size, character of population, industry, and climatic conditions, that they are particularly favorable for purposes of sanitary comparison. Three cities more similar in almost every respect could hardly be found elsewhere. In one important

[1] The merit of this method as compared with certain others in vogue is discussed in Twelfth Census, *Bull. No. 135*, "Methods of Estimating Population."

particular, however, they have differed—viz., in their water-supplies—and, as we shall see below, this fact, in connection with their close similarity in other respects makes their experience almost equivalent to a laboratory experiment. Lawrence, previous to

TABLE 6.

Total Deaths, Deaths from Certain Diseases, and Population, Lawrence, Mass., 1883 to 1905 Inclusive.*

Year	Population	Total Deaths	Typhoid Fever	Pneumonia	Bronchitis	Pulmonary Tuberculosis	Cholera Infantum	Diphtheria and Croup	Apoplexy	Inanition, Marasmus, and Infantile Debility	Heart Disease	Old Age	Diseases of the Kidneys
Merrimac River, unpurified:													
1880	†39,151												
1883	38,978	850	28	60	9	147	57						
1884	38,919	892	19	68	13	142	73						
1885	†38,862	774	17	67	17	163	44						
1886	40,020	763	23	45	14	125	55						
1887	41,178	925	47	74	17	132	71						
1888	42,337	928	48	95	16	134	77	11	17	42	54	29	13
1889	43,495	1,116	55	75	10	110	96	170	23	52	65	17	14
1890	†44,654	1,184	60	117	28	114	96	78	19	74	58	14	20
1891	46,156	1,129	55	133	30	91	101	50	34	69	61	13	24
1892	47,658	1,246	50	156	27	95	130	28	30	94	43	20	10
Change: **1893**	49,160	1,184	39	161	15	113	102	23	29	65	38	23	21
Water-supply, filtered:													
1894	50,662	961	25	88	17	88	101	16	34	56	46	23	17
1895	†52,164	1,060	18	109	23	113	100	14	29	Not given			
1896	54,243	1,101	14	127	32	105	103	25	40	47	62	20	20
1897	56,322	1,159	14	138	26	93	124	47	27	52	73	19	19
1898	58,401	1,153	14	86	30	101	119	63	39	64	82	18	22
1899	60,480	1,235	21	119	36	123	111	64	35	87	87	19	25
1900	†62,559	1,276	13	138	43	116	147	35	34	48	102	19	37
1901	64,057	1,151	13	126	34	108	‡	17	40	44	103	12	23
1902	65,555	1,170	15	169	47	107	‡	12	49	32	87	8	21
1903	67,054	1,226	23	127	36	117	‡						
1904	68,552	1,147	11	159	31	111	‡						
1905	†70,050	1,386	15	203	39	107	‡						

* From Massachusetts State Registration Reports, except last four columns, which are from Reports of Lawrence Board of Health. For certain of the less important causes only the period 1888–1902 inclusive has been taken, the spaces for the remaining years being left blank. Total deaths in this and subsequent Tables 9 and 12 are exclusive of stillbirths.

† Census years. U.S. Census on the even decades; State Census at the intermediate fifth year. Other populations interpolated by the "arithmetical" method.

‡ Title discontinued in State Reports.

1893, had taken its water-supply directly from the polluted Merrimac River only nine miles below the sewers of Lowell, but introduced in that year slow sand filtration; Lowell changed in the years 1894 and 1895 from the same polluted river to a pure ground-water; while Manchester had, throughout the period which we shall consider a surface-water supply of good quality.

The statistics for Lawrence for the period which we have studied are given in Tables 6 and 7.

From these figures we have plotted upon diagrams shown in Chart 4 the death-rates from various causes in Lawrence from 1883–1895 inclusive—a period, that is to say, including the year of the sudden change from an unfiltered to a filtered water-supply, and covering the 10 years before, and 12 years after, that event.

TABLE 7.

TOTAL DEATH-RATES, AND DEATH-RATES FROM CERTAIN DISEASES, LAWRENCE, MASS., 1883 TO 1905 INCLUSIVE, PER 100,000 OF POPULATION.*

Year	Total Death-Rate	Typhoid Fever	Pneumonia	Bronchitis	Pulmonary Tuberculosis	Cholera Infantum	Diphtheria and Croup	Apoplexy	Inanition, Marasmus, and Infantile Debility	Heart Disease	Old Age	Diseases of the Kidneys
Merrimac River, unpurified:												
1883	2,181	72	154	23	377	146						
1884	2,292	49	175	33	365	188						
1885	1,992	44	172	44	419	113						
1886	1,907	58	112	35	312	137						
1887	2,246	114	180	41	320	172						
1888	2,192	113	224	38	317	182	26	40	99	128	69	31
1889	2,566	126	172	23	253	221	391	53	120	149	39	32
1890	2,652	134	262	63	255	215	175	43	166	130	31	45
1891	2,444	119	288	65	197	219	108	74	149	132	28	52
1892	2,614	105	327	57	199	273	59	63	197	90	42	21
Change: **1893**	2,408	79	328	31	230	208	47	59	132	77	47	43
Water-supply filtered:												
1894	1,897	49	174	34	174	199	32	67	111	91	45	34
1895	2,032	35	209	44	217	192	27	56	...	...	..	..
1896	2,030	26	234	59	194	190	46	74	87	114	37	37
1897	2,058	25	245	46	165	220	84	48	92	130	34	34
1898	1,974	24	147	51	173	204	108	67	110	141	31	38
1899	2,042	35	197	60	203	184	106	58	144	144	31	41
1900	2,040	21	221	69	185	235	56	54	77	163	30	59
1901	1,797	20	197	53	169	†	27	62	69	161	19	36
1902	1,785	23	258	72	163	†	18	75	49	133	12	32
1903	1,828	34	190	54	175	†						
1904	1,673	16	232	45	162	†						
1905	1,979	21	290	56	153	†						

* Computed from preceding table.

† Title discontinued in reports. Slow sand filter went into operation September, 1893.

The method of plotting is the same as that in the chart for Hamburg, the vertical lines upon which the rates are plotted representing the middle of each year, and the period of the use of unfiltered Merrimac River water being shown by a black bar running to September, 1893, when filtration was introduced. The scale employed is the same for all curves except the lowest; and, in order to save space,

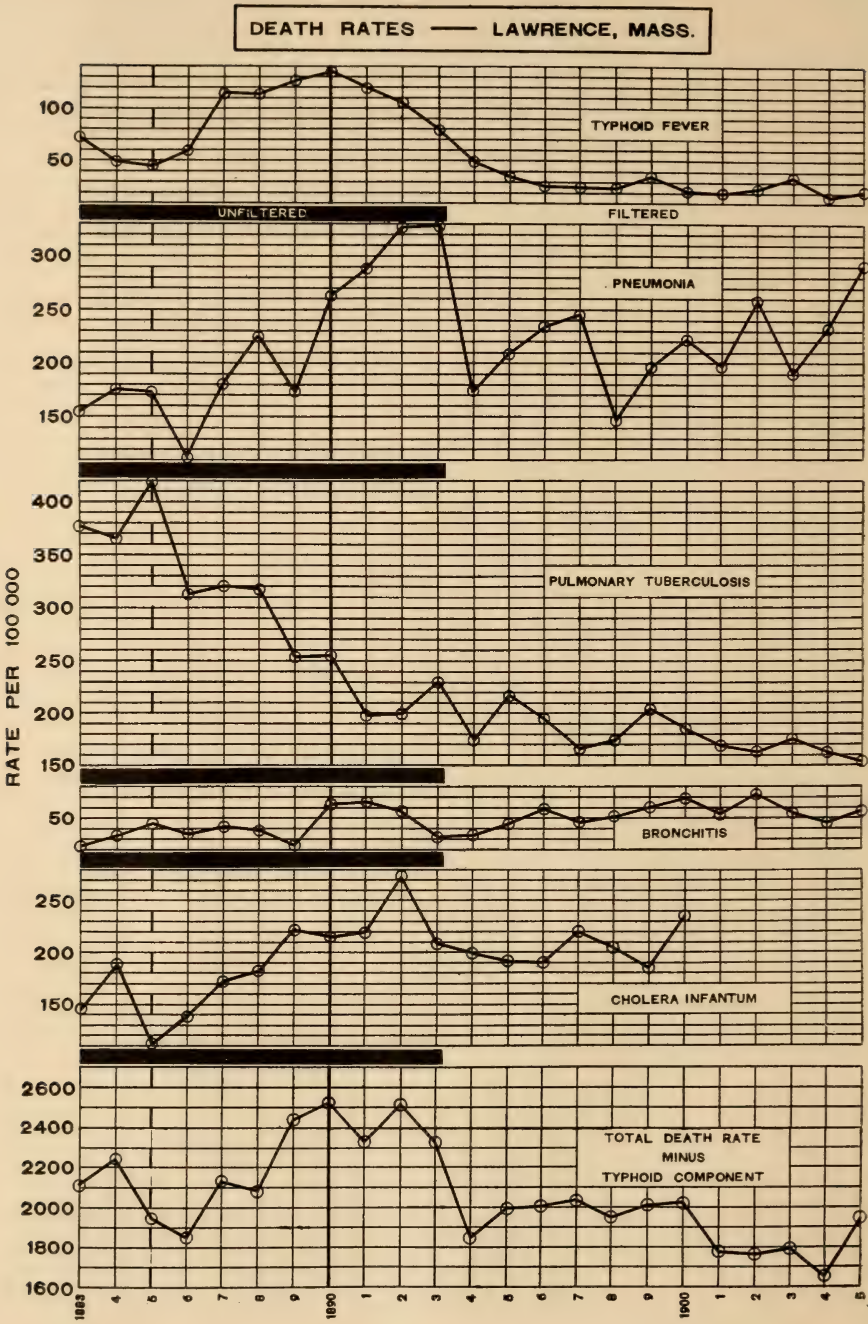
DEATH RATES — LAWRENCE, MASS.
TYPHOID FEVER
UNFILTERED
FILTERED
PNEUMONIA
PULMONARY TUBERCULOSIS
BRONCHITIS
CHOLERA INFANTUM
TOTAL DEATH RATE
MINUS
TYPHOID COMPONENT
RATE PER 100 000
1883
1890
1900

CHART 4.

the unused portion of the scale below each curve has been cut off, so that no base-lines are shown.

If we now proceed to analyze the different curves, we find that typhoid fever, which reached epidemic proportions of large magnitude in 1890, declined even before the introduction of the filter, as might naturally have been expected; but immediately afterward sank lower than for many years, and has since remained on a comparatively low level.

The curve for pneumonia is particularly interesting and striking, for it shows that this disease previous to 1893 had been rising by leaps and bounds most of the time since 1885, and had reached very large proportions in 1893. With the introduction of a purer water-supply pneumonia immediately fell back to a point lower than it had shown since 1889; and, altho it rose again somewhat, remained on a comparatively low average for the next 10 years.

Pulmonary tuberculosis was falling rapidly prior to the introduction of filtration, and continued to fall, tho more slowly, afterward. Altho in the curve any decline in phthisis consequent on water purification is so obscured by the effect of improvement in all sorts of other sanitary conditions that it cannot be directly demonstrated, nevertheless, as we showed in our paper entitled "An Apparent Connection between Polluted Public Water-Supplies and the Mortality from Pulmonary Tuberculosis" (referred to above, p. 491), a distinct effect of the filtration of the water-supply appears in this disease also. The evidence upon which this conclusion is based—as will be seen by reference to the above-mentioned paper, or to sec. xvi of the present paper where the same data are presented—is drawn from an analytical comparison with Manchester (*vide supra*) as a norm.

The curve for bronchitis shows no noteworthy phenomena beyond the lowness of the rates for 1893, 1894, and 1895 as compared with the three years next preceding. For this disease also a comparison with some other city such as Manchester as a control is required and is given beyond.

Cholera infantum, which was quite high for several years before the introduction of filtration, was somewhat lower for a number of years afterward.

When we come to the curve at the bottom of the chart we find a striking demonstration of the Mills-Reincke phenomenon. For we see that during the four years previous to the introduction of filtration the total death-rate had been very high, sometimes a little exceeding and sometimes falling a little below 24 per 1,000, a figure of notable height even if all the typhoid fever deaths had been included. Neglecting the year 1893, inasmuch as this was a year characterized partly by polluted and partly by purified water-supply, and beginning with 1894, we observe a most remarkable and extraordinary drop, a drop all the more surprising and convincing since the death-rate here shown is that from diseases and causes of death other than typhoid fever. This curve shows apparently even more clearly than does the typhoid fever curve itself at the top of the chart the striking effect of the purification of the public water-supply.

XIV. GRAPHICAL DEMONSTRATION AND DISCUSSION OF THE LOWELL PHENOMENA

We now present a graphical demonstration of the phenomena at Lowell, based upon statistics which have the same secure foundation as those for Lawrence.

This city, 28 miles below Manchester, and 12 miles below Nashua, N.H., both the latter cities and several others at greater distances discharging their sewage into the Merrimac River, used the polluted river water without purification until 1893. In that year deep wells were driven and the new water was introduced into the city mains along with the old. The supply of ground-water was gradually increased until 1896, when the river water was entirely shut off. The following table shows the progress of the substitution of pure water for polluted:

TABLE 8.

AVERAGE CONSUMPTION OF RIVER WATER AND OF GROUND-WATER IN LOWELL.*

Year	Total Consumption (Million Gals. per Day)	River Water (Percentage of Total)	Ground-Water (Percentage of Total)
1892	...	100	0
1893	6.8	91	9
1894	6.6	65	35
1895	7.0	33	67
1896	7.0	4	96
1897	...	0	100

* Computed from diagram in *Annual Report of Lowell Water Board, for 1896*. The first well water was pumped September 16, 1893. The river water was finally shut off February 22, 1896. Thus 1893 was essentially the last year of the old supply, and 1896 the first year of the new.

The statistics upon which we have based our studies of Lowell are presented in Tables 9 and 10.

TABLE 9.

TOTAL DEATHS, DEATHS FROM CERTAIN DISEASES, AND POPULATION, LOWELL, MASS., 1883 TO 1905 INCLUSIVE.*

Year	Population	Total Deaths	Typhoid Fever	Pneumonia	Bronchitis	Pulmonary Tuberculosis	Cholera Infantum	Diphtheria and Croup	Apoplexy	Inanition, Marasmus, and Infantile Debility	Heart Disease	Old Age	Diseases of the Kidneys
Merrimac River, unpurified:													
1880	†59,475												
1883	62,253	1,432	49	103	35	249	125						
1884	63,179	1,400	41	114	43	206	136						
1885	†64,107	1,329	49	85	26	196	126						
1886	66,824	1,499	50	106	33	191	165						
1887	69,542	1,869	90	168	64	232	157						
1888	72,260	1,763	62	154	61	190	183	107	34	159	102	31	50
1889	74,978	1,897	69	145	73	213	215	101	39	159	141	55	46
1890	†77,696	1,960	125	145	96	214	218	41	42	164	148	66	56
1891	79,030	1,975	78	187	96	222	251	16	44	155	156	61	66
1892	80,364	2,229	77	215	114	231	229	28	51	231	140	56	64
Years of change:													
1893	81,698	2,108	55	217	126	212	286	36	52	216	128	69	54
1894	83,032	1,790	51	137	90	188	221	36	37	146	123	51	47
1895	†84,367	1,869	33	155	78	160	218	52	59	142	154	62	62
1896	86,487	1,922	38	157	86	185	221	46	51	149	153	68	64
Ground-water:													
1897	88,607	1,860	17	170	107	180	177	40	67	133	158	46	46
1898	90,727	1,809	25	165	100	188	185	37	62	115	163	49	91
1899	92,848	1,851	18	197	75	194	179	41	63	112	156	22	54
1900	†94,969	1,850	19	208	86	181	132	27	77	137	190	40	73
1901	94,953	2,041	18	238	67	160	‡	114	72	156	197	40	82
1902	94,937	1,943	17	189	80	154	‡	73	82	172	219	34	77
1903	94,921	1,899	26	191	71	124	‡						
1904	94,905	1,738	18	177	73	126	‡						
1905	†94,889	1,899	17	178	70	148	‡						

* From Massachusetts State Registration Reports, except last four columns, which are from Reports of Lowell Board of Health. Stillbirths are excluded.

† Census years. U.S. Census on the even decades; State Census at the intermediate fifth year. Other populations interpolated by the "arithmetical" method.

‡ Title discontinued in State Reports. For certain of the less important causes only the period 1888–1902 inclusive has been included, the spaces for the remaining years being left blank.

From these figures the plots in Chart 5 have been constructed. These show the death-rates from various causes for a number of years, as for Lawrence, before and after the purification of the water-supply, including two U.S. Censuses and three state Censuses. Here, as at Lawrence, we have, therefore, a substantial basis for population statistics. The method of plotting is the same as for Hamburg and Lawrence. The gradual substitution of unpolluted for polluted water is represented by the tapering portion of the black bar. The

same scale is used for all curves except the bottommost, and here again the diagrams have been cut off so that no base-lines appear.

TABLE 10.

Total Death-Rates, and Death-Rates from Certain Diseases, Lowell, Mass., 1883 to 1905 Inclusive, per 100,000 of Population.*

Year	Total Death-Rate	Typhoid Fever	Pneumonia	Bronchitis	Pulmonary Tuberculosis	Cholera Infantum	Diphtheria and Croup	Apoplexy	Inanition, Marasmus, and Infantile Debility	Heart Disease	Old Age	Diseases of the Kidneys
Merrimac River, unpurified:												
1883	2,300	79	165	56	400	201						
1884	2,216	65	180	68	326	215						
1885	2,073	76	133	41	306	196						
1886	2,243	75	159	49	286	247						
1887	2,688	129	242	92	334	226						
1888	2,440	86	213	84	263	253	148	47	220	141	43	69
1889	2,530	92	193	97	284	287	135	52	212	188	73	61
1890	2,523	161	187	123	276	280	53	54	211	190	85	72
1891	2,499	99	237	121	281	318	20	56	196	197	77	84
1892	2,774	96	268	142	288	285	35	64	287	174	70	80
Years of change:												
1893	2,580	67	266	154	259	350	44	64	264	157	84	66
1894	2,156	61	165	108	226	266	43	45	176	148	61	57
1895	2,215	39	184	92	190	258	62	70	168	183	73	73
1896	2,222	44	182	99	214	256	53	59	172	177	79	74
Ground-water:												
1897	2,099	19	192	121	203	200	45	76	150	178	52	52
1898	1,994	28	182	110	207	204	41	68	127	180	54	100
1899	1,994	19	212	81	209	193	44	68	121	168	24	58
1900	1,948	20	219	91	191	139	28	81	144	200	42	77
1901	2,150	19	251	71	168	‡	120	76	164	207	42	86
1902	2,047	18	199	84	162	‡	77	86	181	231	36	81
1903	2,001	27†	201	75	131	‡						
1904	1,831	19	187	77	133	‡						
1905	2,001	18	188	74	156	‡						

* Computed from preceding table.

† Epidemic August, 1903, due to check-valve accident.

‡ Title discontinued in reports.

Changed from raw river water to deep wells. First ground-water pumped September, 1893. River water finally shut off February, 1896.

Beginning with the curve for typhoid fever, we find that here, as at Lawrence, the mortality had been subsiding, as was to be expected after the epidemic proportions of 1890; but sank still lower in the years of introduction of ground-water, remaining since at a comparatively low level.

Pneumonia, which had been on the whole rising, distinctly tho irregularly for many years, exhibits the striking phenomenon of an immediate drop from the high point which it had reached in 1893. The comparatively low level to which it now descended was, moreover,

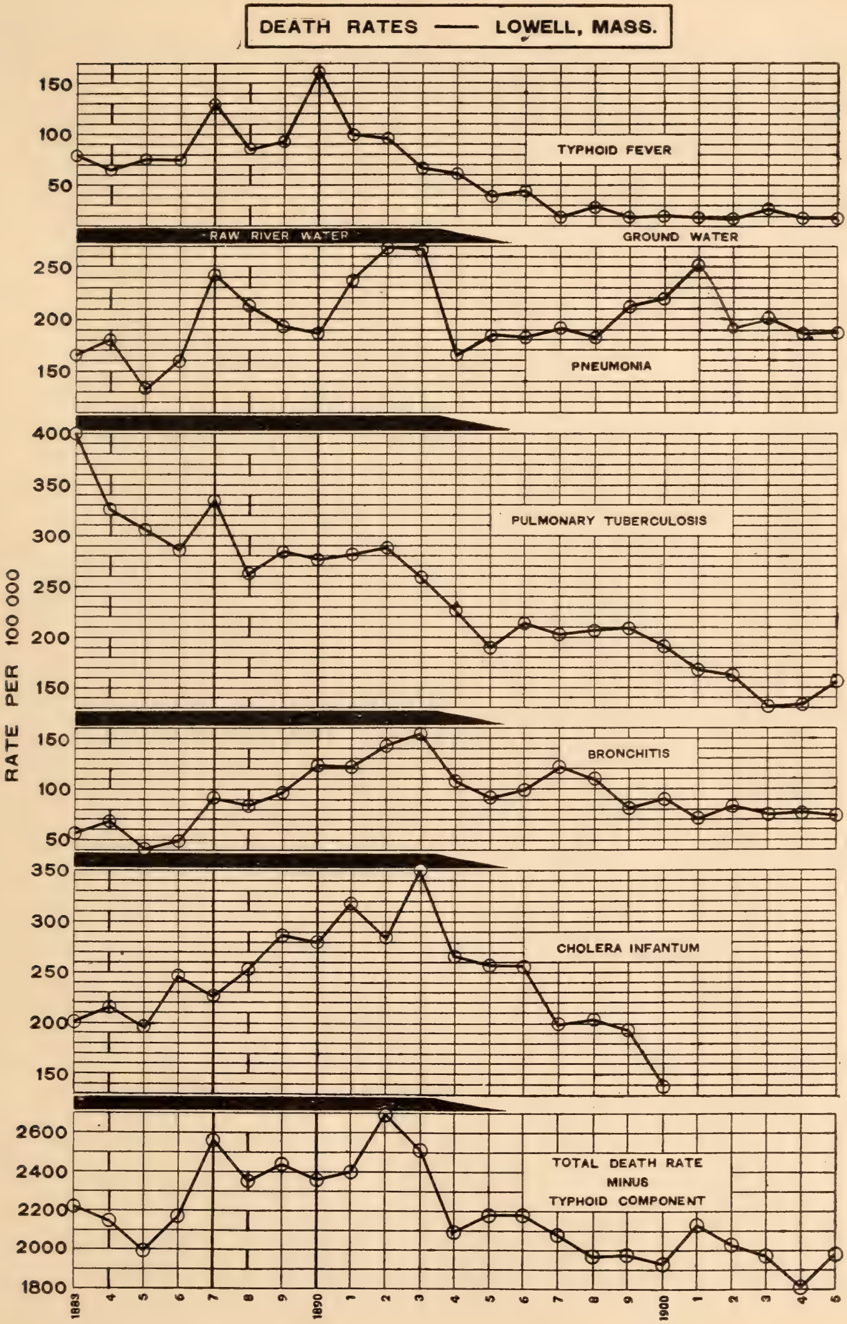
DEATH RATES —— LOWELL, MASS.
TYPHOID FEVER
RAW RIVER WATER
GROUND WATER
PNEUMONIA
PULMONARY TUBERCULOSIS
BRONCHITIS
CHOLERA INFANTUM
TOTAL DEATH RATE
MINUS
TYPHOID COMPONENT
RATE PER 100 000
1883
1890
1900

Chart 5.

in general maintained, tho with certain fluctuations, up through the last year shown.

Pulmonary tuberculosis had been declining steadily prior to 1894, and from the curve no influence of purification of water-supply can be demonstrated, but that in this disease also the benefits of the better water were felt is clearly indicated by the analytical study in our contribution to the volume "Tuberculosis in Massachusetts" (referred to, p. 491 above) and by later study in sec. xvi of the present paper.

Bronchitis had been rising rapidly, up to the critical years 1894 and 1895, but at this time the whole trend of the curve was reversed, a distinct fall setting in—more rapid at first, and later settling gradually to the comparatively low level maintained for the last seven years of the period.

One of the most striking effects is seen in cholera infantum, which had been on the increase for some years before introduction of groundwater, but immediately afterward showed a rapid descent. Unfortunately reliable data for 1901 ff. were not available, but these years are less important than the years immediately following purification.

Finally, we discover, as at Lawrence, in the last curve of the chart an extraordinary demonstration of the Mills-Reincke phenomenon. The total death-rate minus typhoid component, which had risen as high as 27 and had scarcely gone below the excessive rate of 24 in the seven years prior to the critical years 1894–95, now shows in 1894 the surprising drop to a level of 21, sinking in subsequent years even lower.

XV. A GRAPHICAL DEMONSTRATION OF THE PHENOMENA AT MANCHESTER, NEW HAMPSHIRE, A CITY HAVING A WATER-SUPPLY OF GOOD QUALITY.

As a control or norm with which the phenomena at Lawrence and Lowell may be compared we have taken Manchester, N.H., a city which, as we have already stated, is in all important respects closely similar to Lawrence and Lowell. This similarity may be demonstrated statistically by reference to the U.S. Census for 1890 and 1900 and the Massachusetts State Census for 1895. We have taken from those sources figures relating to age, sex, and nativity distribu-

tion, and occupations of the population, which not only demonstrate the similarity of the three cities in the respects stated but also the fact that the three censuses show no significant differences in the percentages. It is a well-known fact that the mortality rates of communities may be considerably affected by the age distribution of the population, since death-rates are much higher for the very young and the very old. The death-rates of the age-groups under five years and over forty-five years are usually higher than that of the whole population, whereas the death-rates of other ages are lower, tho the limits may vary somewhat for different cities. By reference, for instance, to the Massachusetts State Registration Report for 1895, we ascertain the death-rate for that year for the whole population to be 19.01 per 1,000; the rate for the age-group under five years, 64; for the group five–ten, 6; for succeeding age-groups up to fifty, rates less than 19; but for all groups above fifty, rates higher again than that for the whole population. Again, as is well known, general death-rates are affected to greater or less extents by distribution according to sex, place of birth, and occupations of the population. We therefore present without further comment the following table:

TABLE 11.*

Ratios of:	Percentages							
	Lawrence			Lowell			Manchester	
	1890	1895	1900	1890	1895	1900	1890	1900
Age-groups under five and over forty-five to total population	28.0	28.2	28.6	27.4	27.9	28.4	27.2	29.1
Male population to total population	46.6	48.2	48.3	45.6	46.1	47.3	45.0	46.7
Persons born in Canada (English) to total population	10.0†	2.0	2.7	20.3†	1.9	4.7	28.1†	3.0
Persons born in Canada (French) to total population		8.9	11.2		15.2	15.4		23.6
Persons born in Great Britain‡ to total population	13.7	12.9	10.2	6.6	6.7	5.9	3.5	3.1
Persons born in Germany to total population	4.1	4.6	3.9	0.2	0.2	0.2	2.0	2.0
Persons born in Ireland to total population	17.2	14.4	11.3	16.3	14.9	12.8	9.8	7.4
Persons born in other countries to total population	0.9	3.8	6.4	1.1	5.3	4.1	2.1	3.5
Total foreign-born	45.9	46.6	45.7	44.5	44.2	43.1	45.5	42.6
Wage-earners to total population	34.9	....	35.7	35.7	....	33.0	34.8	33.4
Textile operatives¶ to total wage-earners	68.9	....	76.1	63.7	....	65.0	62.2	58.7

* Computed from U.S. Censuses, 1890 and 1900, and Massachusetts Census, 1895.

† Canada and Newfoundland.

‡ England, Scotland, and Wales.

¶ Workers in manufacture of cotton, woolen, knit, and worsted goods, and hosiery.

Thus the above statistics confirm and supplement the knowledge gained by the personal familiarity of the authors with these three cities that they are in all important respects closely similar.

The statistics upon which we have based our studies of Manchester are presented in Tables 12 and 13.

TABLE 12.

TOTAL DEATHS, DEATHS FROM CERTAIN DISEASES, AND POPULATION, MANCHESTER, N.H., 1883 TO 1905 INCLUSIVE.*

Year	Population	Total Deaths	Typhoid Fever	Pneumonia	Bronchitis	Pulmonary Tuberculosis	Cholera Infantum	Diphtheria and Croup	Apoplexy	Inanition, Marasmus, and Infantile Debility	Heart Disease†	Old Age	Nephritis, incl. Bright's Disease
Same water-supply, of good quality, throughout:													
1880	‡32,630												
1883¶	35 220	760	20	54	7	116	131						
1884	37,230	764	17	46	18	117	112						
1885	38,380	783	22	55	20	107	88						
1886	39,530	748	15	33	9	118	122						
1887	40,680	814	18	51	31	90	148						
1888	41,830	869	14	55	37	115	117	55	11	31	46	24	5
1889	42,980	778	18	53	35	78	86	49	18	22	47	18	12
1890	‡44,126	973	15	83	40	106	146	28	18	36	43	27	22
1891	45,410	895	19	85	38	98	122	9	15	24	42	18	17
1892	46,700	980	5	50	38	91	102	11	21	20	56	20	24
1893	47,980	1,013	14	66	43	91	86	7	23	21	44	28	13
1894	49,270	1,001	23	86	59	92	138	37	15	38	55	13	13
1895	50,560	1,036	21	107	55	83	144	26	19	47	80	22	16
1896	51,840	1,064	19	90	54	126	115	45	17	29	77	21	21
1807	53,130	1,140	12	119	88	111	123	38	29	39	70	18	22
1898	54,410	1,003	14	89	47	88	136	26	26	33	53	15	31
1899	55,700	1,061	11	134	65	101	92	13	31	61	69	21	31
1900	‡56,987	1,153	9	120	39	117	148	13	34	56	84	21	30
1901	58,270	1,143	9	115	37	104	132	8	27	35	88	13	36
1902	59,560	1,088	8	121	55	78	§	30	38	§	72	20	28
1903	60,850	1,112	9	112	41	79	§						
1904	62,130	1,015	12	101	31	87	§						
1905	63,420	1,339	11	129	22	108	§						

* From N.H. State Registration Reports, except last four columns, which were kindly furnished by Dr. Irving A. Watson, State Registrar of Vital Statistics, N.H. For certain of the less important causes only the period 1888–1902 inclusive has been included, the spaces for the remaining years being left blank. Stillbirths are excluded.

† Including angina pectoris, embolism, and phlebitis.

‡ U.S. Census years. Intermediate populations interpolated by the "arithmetical" method.

¶ Year ending March 31, 1883.

§ Change of classification.

From these death-rates we have constructed the diagrams in Chart 6, using the same method of plotting as for Hamburg, Lawrence, and Lowell. As Manchester had a water-supply of good quality throughout, there are no black bars.

Typhoid fever remained at a fairly low level throughout the period, and this was to be expected from the character of the water-supply. This curve therefore affords a valuable background for viewing the typhoid fever death-rates of the other two cities.

TABLE 13.

TOTAL DEATH-RATES, AND DEATH-RATES FROM CERTAIN DISEASES, MANCHESTER, N.H., 1883 TO 1905 INCLUSIVE, PER 100,000 OF POPULATION.*

Year	Total Death-Rate	Typhoid Fever	Pneumonia	Bronchitis	Pulmonary Tuberculosis	Cholera Infantum	Diphtheria and Croup	Apoplexy	Inanition, Marasmus, and Infantile Debility	Heart Disease†	Old Age	Nephritis, incl. Bright's Disease
1883	‡2,158	57	153	20	329	372						
1884	2,052	46	124	48	314	301						
1885	2,040	57	143	52	279	229						
1886	1,892	38	84	23	299	309						
1887	2,001	44	125	76	221	364						
1888	2,077	33	132	88	275	280	132	26	74	110	57	12
1889	1,810	42	123	81	181	200	114	42	51	109	42	28
1890	2,205	34	188	91	240	331	63	41	82	97	61	50
1891	1,971	42	187	84	216	269	20	33	53	93	40	37
1892	2,099	11	107	81	195	219	24	45	43	120	43	51
1893	2,111	29	138	90	190	179	15	48	44	92	58	27
1894	2,032	47	175	120	187	280	75	30	77	112	26	26
1895	2,049	42	212	109	164	285	51	38	93	158	44	32
1896	2,052	37	174	104	243	222	87	33	56	149	41	41
1897	2,146	23	224	166	209	232	72	55	73	132	34	41
1898	1,842	26	164	86	162	250	48	48	61	97	28	57
1899	1,905	20	240	117	181	165	23	56	109	124	38	56
1900	2,023	16	210	68	205	260	23	60	98	147	37	53
1901	1,962	15	197	64	179	227	14	46	60	151	22	62
1902	1,827	13	203	92	131	...	50	64	...	121	34	47
1903	1,827	15	184	67	130	...						
1904	1,634	19	163	50	140	...						
1905	2,111	17	204	35	170	...						

* Computed from preceding table.

† Including angina pectoris, embolism, and phlebitis.

‡ Year ending March 31, 1883.

Same surface water-supply, of good quality, throughout.

Pneumonia was rising steadily tho irregularly until 1899, and at the very time (1894) when Lawrence experienced an extraordinary drop in pneumonia and Lowell one nearly as great, the rate for Manchester had risen higher than it had ever been before in the period considered; and then while the rates at Lawrence and Lowell remained at the level to which they had fallen, that at Manchester, on the whole, rose.

For pulmonary tuberculosis the Manchester curve, like those for the other two cities, was throughout the period falling gradually,

but its rate of fall was in comparison not so rapid, the years 1896 and 1897 even showing a marked rise.

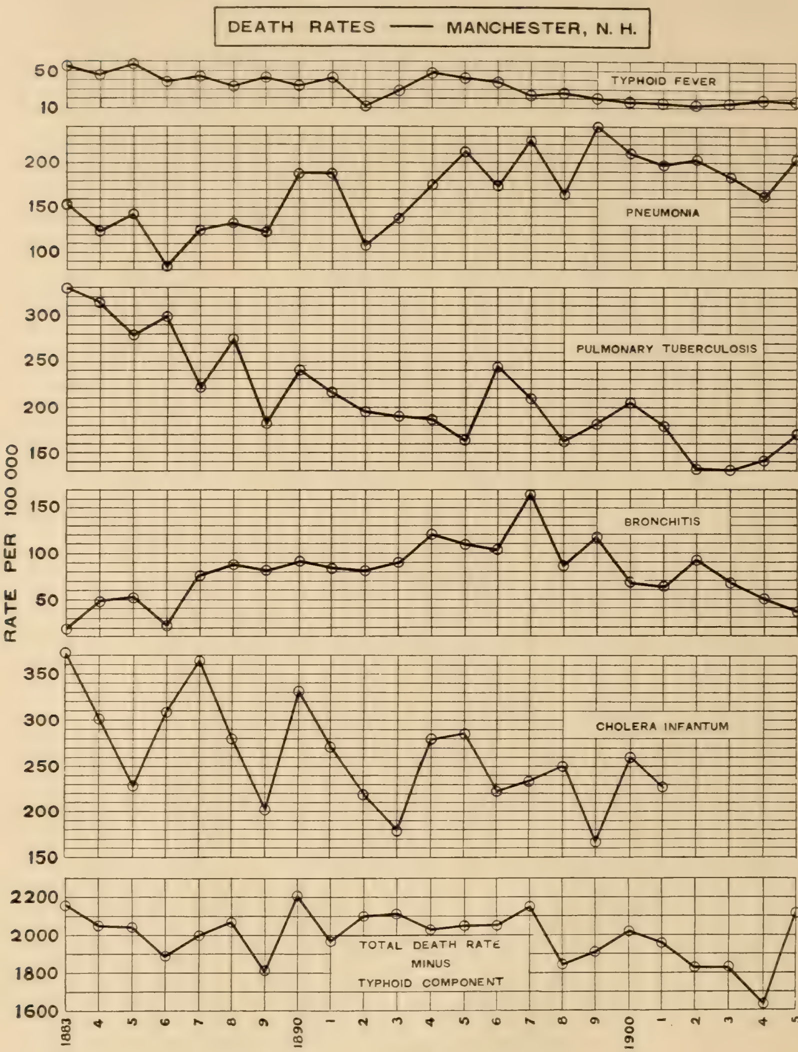

CHART 6.

Bronchitis, like pneumonia, was rising at Manchester until 1897, while at Lowell there was a decline in this disease following the introduction of ground-water. At Lawrence the decrease in bron-

chitis had already set in before filtration, hence without the further detailed studies which we present in sec. xvi, the fact that Lawrence experienced a greater decline than the norm cannot be readily seen.

In cholera infantum the curve for Manchester is highly irregular, but in the years 1894 and 1895, when that disease had dropped markedly in the other two cities, the rate for Manchester had risen to a maximum.

In the curve for total death-rate minus typhoid component Manchester shows a close adherence to a level of about 2,000 from 1892 to 1897, thus covering the period when Lawrence and Lowell changed their water-supplies and experienced remarkable drops in that death-rate, which had in these two cities been at a much higher level.

We shall not pause here any longer to discuss Lawrence and Lowell as compared with Manchester, reserving for sec. xvi the detailed study of the comparative relations among these three cities.

XVI. A COMPARATIVE STUDY OF THE DATA DERIVED FROM LAWRENCE, LOWELL, AND MANCHESTER.

We have seen in secs. xiii, xiv, and xv that both at Lawrence and at Lowell there followed immediately upon purification of the water-supply a lasting drop in the total death-rate—a drop much greater than could be accounted for by the decrease in typhoid fever alone; while the total death-rate at Manchester showed no such change, but kept steadily on the same comparatively low level.

The drops at Lawrence and Lowell we shall examine somewhat more closely in order to determine: (1) the extent of the reductions in their total death-rates in comparison with the norm, Manchester; and (2) what causes of death played the principal parts in these reductions. Throughout we shall compare Lawrence and Lowell with each other and with the norm, Manchester.

In the analysis of the declines we have first taken seven chief causes of death, omitting "accident," as given in the tables of the Massachusetts State Registration Report for 1890. These are: phthisis, causing in that year 5,791 deaths; pneumonia, 4,038; cholera infantum, 2,491; diphtheria and croup, 1,626; bronchitis, 1,533; apoplexy, 1,301; and typhoid fever, 835; making a total of 17,615 deaths. The total number of deaths in Massachusetts for

the year was 43,528. In addition to this list we have taken several important general headings given in most nosological arrangements, in order to include diseases of all the general classes, viz.: inanition, marasmus, and infantile debility; heart disease; old age; and diseases of the kidneys. The sum of all titles above mentioned formed 50 to 60 per cent of the total deaths in Manchester, Lowell, and Lawrence, and if causes of death other than disease be omitted, the percentage is somewhat higher.

It may be objected that a few of these titles, e.g., old age, are too vague to serve in an accurate analysis, and the objection is valid so far as exact and absolute determinations of mortality are concerned. For purposes of comparison of the rates in the same city at periods not much separated, however, the indefiniteness of any given title does not greatly affect its value in the analysis. The limits covered by any title depend upon the local usage of the medical profession in the community in question; and as such usage commonly changes only gradually, year to year comparisons are not seriously interfered with. The important phenomena considered in this paper are *sudden* decreases in death-rates taking place within a short space of time.

Mortality statistics for the various causes of death, illustrating the phenomena at Lawrence and Lowell, as compared with the norm, Manchester, before and after water-supply purification, are given in Tables 14 and 15. By the use of averages for periods of five years before and after, a safer if less striking view of the facts is obtained.

From the first of these tables have been plotted Charts 7 and 8, which largely explain themselves. It must be observed that the periods of five years each, before and after water-supply purification, do not quite correspond for Lawrence and Lowell, so that the corresponding Manchester blocks in the upper and lower halves of the chart do not represent exactly the same periods for that city.

These diagrams demonstrate certain points which do not clearly appear in the curves in Charts 4 and 5, one of the chief advantages being that the movements of the rates in Manchester, the norm, are readily seen in direct comparison with those in the other two cities. Thus, for example, the stationary bronchitis rate at Lawrence is seen to be actually a gain because that at Manchester increased; a point which does not appear in the Lawrence curve alone.

In order that the actual extent of the decreases in death-rates may be determined, reckoned against the corresponding changes at

TABLE 14.

MORTALITY RATES FROM CERTAIN DISEASES IN LAWRENCE, MASS. (AS COMPARED WITH MANCHESTER, N.H.), BEFORE AND AFTER WATER-SUPPLY PURIFICATION.

Mean annual death-rates (per 100,000) for five-year periods just before, and just after, introduction of filtration at Lawrence.

	Lawrence		Manchester	
	1888–1892 (Before)	1894–1898 (After)	1888–1892 (Same Periods as for Lawrence)	1894–1898
Typhoid fever	119	32	32	35
Pneumonia	255	202	148	190
Bronchitis	49	47	85	117
Pulmonary tuberculosis	244	185	222	192
Cholera infantum	222	201	260	254
Diphtheria and croup	152*	59	71	67
Apoplexy	55	62	37	41
Inanition, marasmus, and infantile debility	146	100	61	72
Heart disease	126	119	106	130
Old age	42	37	49	35
Diseases of the kidneys	36	36	36	39
Total death-rate	2,494	1,998	2,032	2,024

* Epidemic in 1889, when the rate rose to 391.

TABLE 15.

MORTALITY RATES FROM CERTAIN DISEASES IN LOWELL, MASS. (AS COMPARED WITH MANCHESTER, N.H.), BEFORE AND AFTER WATER-SUPPLY PURIFICATION.

Mean annual death-rates (per 100,000) for five-year periods just before, and just after, introduction of ground-water at Lowell.

	Lowell		Manchester	
	1889–1893 (Before)	1896–1900 (After)	1889–1893 (Same Periods as for Lowell)	1896–1900
Typhoid fever	103	26	32	24
Pneumonia	230	197	149	202
Bronchitis	127	100	85	108
Pulmonary tuberculosis	278	205	205	200
Cholera infantum	304	198	240	226
Diphtheria and croup	57	42	47	51
Apoplexy	58	70	42	50
Inanition, marasmus, and infantile debility	234	143	55	79
Heart disease	181	181	102	130
Old age	78	50	49	36
Diseases of the kidneys	73	72	39	50
Total death-rate	2,581	2,051	2,039	1,994

Manchester, as well as independently, we have also made the computations presented in Table 16.

From these figures it is seen that when the decreases in Manchester are taken into account, and subtracted algebraically with the proper

signs, as normal, from those in the other two cities, the declines in certain diseases in the latter cities stand out from the obscurity

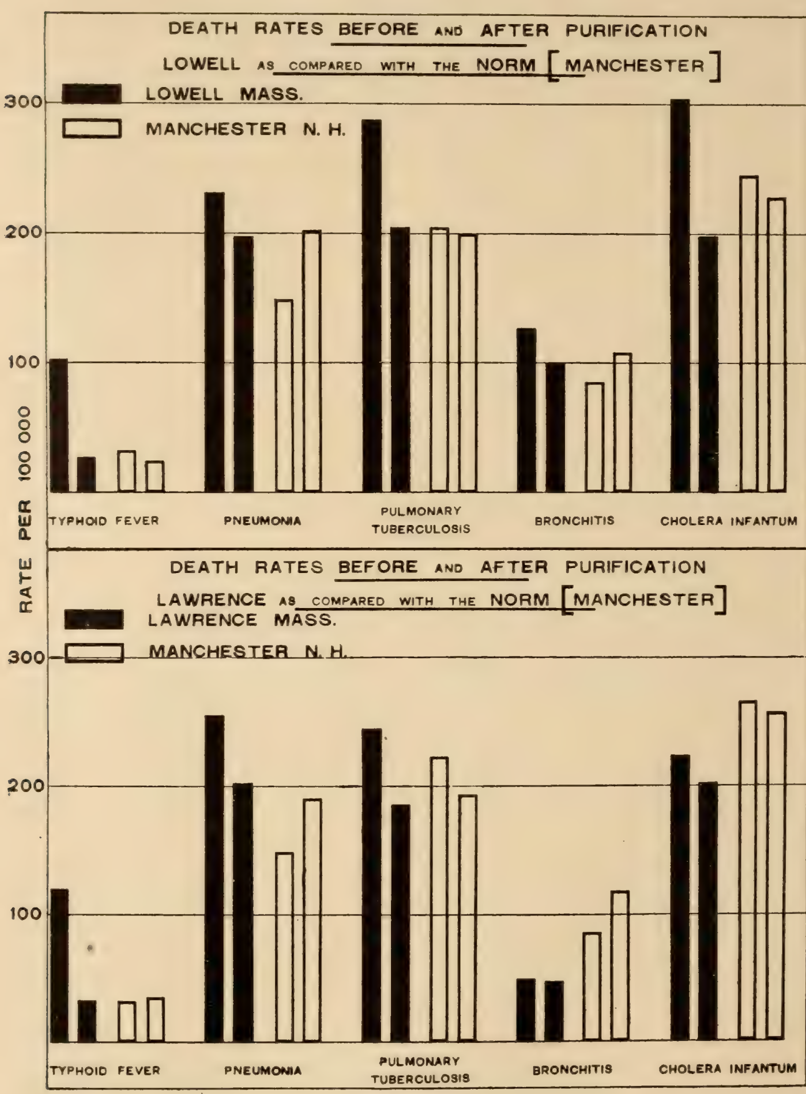

CHART 7.

in which they are masked when only the actual decreases are considered. Chief among these diseases are pneumonia and bronchitis.

Taking up first typhoid fever, and referring to Chart 7 as well as to the tables just given, we find that the rates at Manchester from that disease show the good and unvarying quality of the water-supply there, thus demonstrating the city's normality in that respect. The great drops at the other two cities following water-supply purification stand out clearly.

TABLE 16.

DECREASES IN DEATH-RATES FROM CERTAIN CAUSES IN LAWRENCE AND LOWELL FOLLOWING WATER-SUPPLY PURIFICATION.

Rates are per 100,000 population.

Causes of Death	Decreases in Death-Rates							
	Apparent (Computed Directly)				True (Subtracting Algebraic Decrease at Manchester as Normal)			
	Lawrence		Lowell		Lawrence		Lowell	
	Decrease	Percentage of Total Decrease	Decrease	Percentage of Total Decrease	Decrease	Percentage of Total Decrease	Decrease	Percentage of Total Decrease
Typhoid fever	87	18	77	15	90	18.5	69	14
Pneumonia	53	11	33	6	95	19.5	86	18
Bronchitis	2	0	27	5	34	7	50	10
Pulmonary tuberculosis	59	12	73	14	30	6	68	14
Cholera infantum	21	4	106	20	15	3	92	19
Diphtheria and croup	93	19	15	3	89	18	19	4
Inanition, marasmus, and infantile debility	46	9	91	17	57	12	115	24
Old age	5	1	28	5	−9	−2	15	3
Other causes	130	26	80	15	87	18	−29	−6
All causes	496	100	530	100	488	100	485	100

In respect to pneumonia, while Manchester rose markedly from 148 to 190, Lawrence dropped from the high rate of 255 to the normal of 202. Lowell shows a similar decrease. In both cases the closeness of the rates, after improvement, to those of Manchester is additional evidence for the conclusion that the polluted water had caused an abnormally high rate, which declined at once, on the removal of the pollution, to the normal level.

Pulmonary tuberculosis was decreasing in Massachusetts and New Hampshire during the period 1890–1900. The diminution for the state of Massachusetts—shared fully, as detailed study shows, by cities of the same class as Lawrence and Lowell—is indicated by the following rates per 100,000 from the state registration: for 1890,

259; for 1895, 219; and for 1900, 185. Similarly in New Hampshire the rates were: for 1890, 219; for 1895, 177; and for 1900, 158. In a comparison of Manchester and Lawrence, both cities show a decrease, but that in Manchester is comparatively small. The decrease in the latter is what might be expected from general improvement in sanitary conditions. Lawrence starting higher came down to about the level of the norm. Lowell shows the same phenomenon as compared with Manchester, but in much more marked degree. In actual figures Lawrence shows a decrease 30 greater than the normal, and Lowell, 68 greater. These facts, in spite of many complicating factors, indicate a certain amount of decrease due to the change in water-supply. Phthisis being a disease of slow development and often long-postponed fatality, an effect on the mortality could hardly be expected to appear immediately after an improvement in one condition favoring its spread or fatality; and where many such conditions are involved the effect might well be somewhat obscured. We wish therefore to emphasize particularly the evidence here given that both at Lawrence and at Lowell there were decreases in pulmonary tuberculosis following water-supply purification considerably greater than the corresponding normal decreases at Manchester, as this point is not distinctly demonstrable by the curves for the former cities alone.

Bronchitis, which includes both chronic and acute disease, shows much the same phenomena as does pneumonia. At Manchester the rate rose distinctly; at Lawrence, however, it did not rise, but remained stationary; while at Lowell there was actually a decrease amounting to 50 more than the normal decrease. This also is a case of improvement which is much better demonstrated by the blocks of Chart 7 than by the curves.

Cholera infantum, a general title indicative of intestinal diseases of infants, shows a decrease in all three cities. In Lawrence, however, there was a decrease of 15 more than the normal, while Lowell shows a remarkable corresponding surplus decrease of 92. The latter stands out as one of the most striking phenomena observed, and recalls with emphasis Dr. Reincke's statement that the mixing of raw river water with milk caused much of the infant mortality at Hamburg (pp. 497, 500, 502, above). The same

explanation probably also holds good to a very great extent for Lawrence and Lowell.

Among the minor causes of death not plotted in the charts, tho included in the tables, we consider first inanition, marasmus, and infantile debility, a heterogeneous and indefinite title, but one fairly indicative of the mortality of the feeble. Infantile debility makes up a large part of it, and probably a considerable number of deaths from diarrhea and the gastro-intestinal diseases of infants are here included through false diagnosis. As seen from the tables, both at Lawrence and at Lowell the mortality shows a great decrease, while at Manchester a considerable rise took place. On account of the tendency in diagnosis just mentioned it well may be that much of the effect of water-supply purification at Lawrence upon infant mortality is indicated by this title, the cholera infantum rate not having been much lowered.

For old age the figures indicate a slight rise at Lawrence, but at Lowell a decrease distinctly greater than the normal.

Heart disease (uncertain in diagnosis) shows improvements greater than the normal by about 30.

In diseases of the kidneys and apoplexy there are no phenomena worthy of mention.

With reference to diphtheria and croup the mixt character of the title, the highly epidemic character of diphtheria, and the use of antitoxin complicate matters so much that we can draw no conclusions from the figures.

The Mills-Reincke phenomenon at both Lawrence and Lowell is shown very strikingly by Chart 8. (We must caution the reader, that in order to save space the blocks in this chart have been cut off so that their true bases do not appear.) Here the drops seen in the curves in Charts 4 and 5 are reduced to actual quantitative statement for five-year periods just before and after water-supply purification. At Lawrence the total death-rate minus typhoid component dropped from 23.7 to 19.7 per 1,000 with an annual saving of about 200 lives from causes other than typhoid fever. At Lowell it dropped from 24.8 to 20.3, with a saving of 365 lives from such causes. Manchester, on the other hand, shows decreases for the corresponding periods of only 0.1 and 0.4 respectively. In other words, the

REDUCTION IN DEATH RATE

FROM

DISEASES OTHER THAN TYPHOID FEVER

FOLLOWING

WATER SUPPLY PURIFICATION

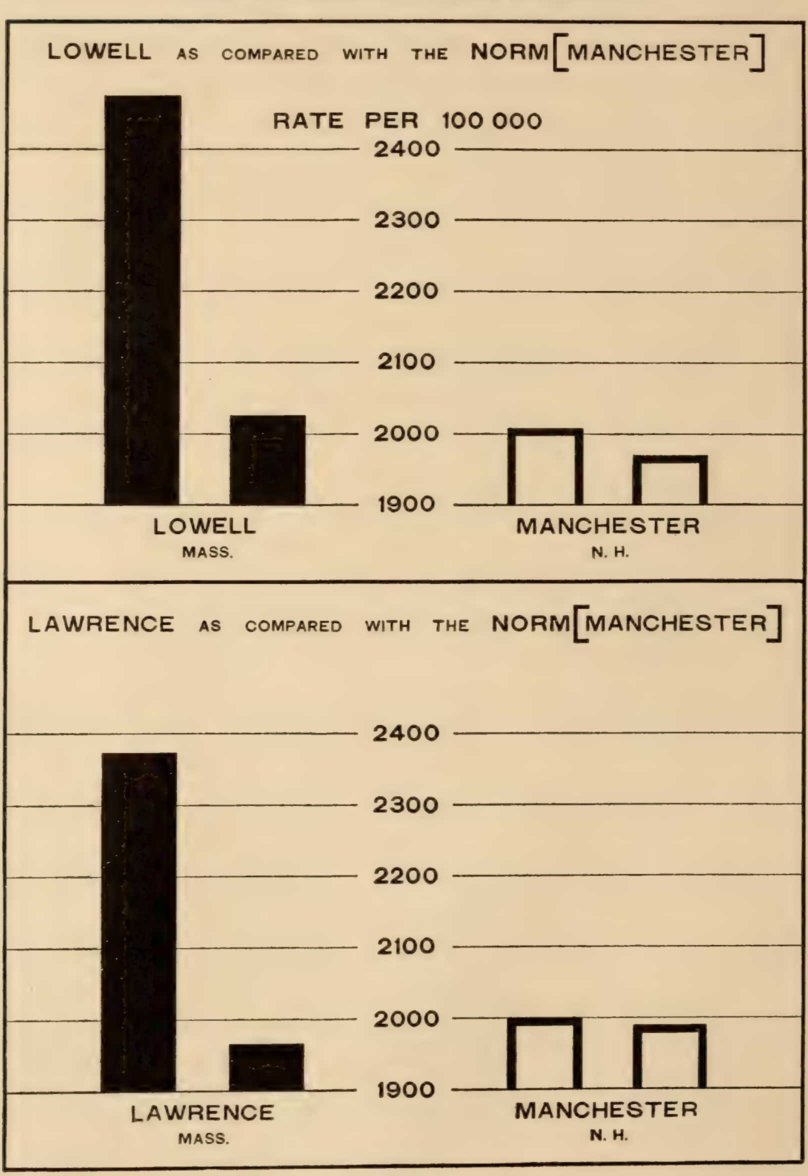

Chart 8.

first two cities, which had used polluted and unpurified water-supplies and had had very excessive "total minus typhoid" death-rates, dropped after purification to the normal and comparatively low rate level (*ca.* 20.0) which Manchester had maintained throughout.

The second of the two questions proposed at the outset of this section, i.e., what causes of death played the principal parts in the reductions in the total death-rates, is also answered in Table 16 above, by the percentage columns. If the rates for Lawrence and Lowell be averaged, over 80 per cent of the decreases in total death-rates—whether actual or comparative (against Manchester) rates be considered—are accounted for. In this decrease, the chief parts were played (besides typhoid fever) by pneumonia, pulmonary tuberculosis, cholera infantum, and inanition, marasmus, and infantile debility. Cholera infantum and the last three causes of death are, as we have already stated, somewhat related by diagnosis. And these results agree with those already obtained for Hamburg (secs. iv and v).

The foregoing analysis affords a basis for a testing of Hazen's theorem. As a preliminary computation we find that at Lawrence, for every death less from typhoid fever there were 4.7 deaths fewer from other causes, and at Lowell 5.9 fewer. These, however, are only crude ratios which include any rises or falls of the rates which would have taken place in any event independent of water-supply purification. It is therefore necessary, in order to obtain ratios comparable with that suggested by Mr. Hazen (which was based upon computations from which decreases due to causes other than water-supply purification had been approximately eliminated), to consider the decreases *minus* those at Manchester as normal. We compute, therefore, from Table 16, that—

> Where one death from typhoid fever has been avoided by the use of better water, a certain number of deaths [*4.4 at Lawrence and 6.0 at Lowell*] from other causes have been avoided.

Thus Mr. Hazen's estimate of two or three is seen to have been for these cities very conservative.

At this point we may briefly consider certain objections which may perhaps be raised against the accuracy of the foregoing statistical studies or our interpretation of them. We have already shown (sec.

iv) that for three census years (1890, 1895, and 1900) the age and sex distributions, the proportions of foreign-born of various nationalities, and the occupations of the population at Lawrence and Lowell were practically the same. Between census years there were undoubtedly fluctuations in these conditions, but any such could have been only temporary, as otherwise they would be indicated by the census figures. Of fluctuations in the actual number of the total population as related to population estimates we have made a special study, the results of which are submitted in sec. xviii. Anticipating, we may state here our conclusion that no error seriously affecting our results has been introduced from that direction. Moreover, important fluctuations in quality or quantity of population at Lawrence and Lowell should have had a counterpart at Manchester, a city in all essential respects similar, with which comparisons have throughout been made. The same remark applies also to mortality decreases due to general sanitary and medical improvement (upon which, it will be remembered, Dr. Reincke laid considerable stress in the case of Hamburg).

Such *sudden* and *lasting* decreases in death-rates as took place in each of these cities can be explained only by sudden and permanent measures of sanitation, and it is impossible to escape the conclusion that the principal part in the drop in total death-rate—the Mills-Reincke phenomenon—both at Lawrence and at Lowell, was played by the purification of the public water-supply.

XVII. STUDIES OF SIMILAR PHENOMENA IN SOME OTHER AMERICAN CITIES

1. *Albany, N.Y.* — As another example of the Mills-Reincke phenomenon occurring in a prominent city which has purified its public water-supply and for which reliable vital statistics are obtainable, we present Albany, N.Y.

Previous to 1899 two-thirds of the total water-supply of this city was taken from the Hudson River without purification, while the remaining third came by gravity from watersheds just west of the city. In September, 1899, slow sand filtration of the Hudson River supply was introduced, while the surface water-supply remained unchanged. The statistics upon which we have based our demonstration of the Albany phenomena are given in the following Tables 17 and 18.

The death-rates for Albany are plotted on Chart 9, in which the same methods of presentation are employed as for Hamburg, Lawrence, and Lowell.

TABLE 17.

TOTAL DEATHS, DEATHS FROM CERTAIN DISEASES, AND POPULATION, ALBANY, N.Y., 1885–1907 INCLUSIVE.*

Year	Population	Total Deaths	Typhoid Fever	Acute Respiratory Diseases	Pulmonary Tuberculosis	Diarrheal Diseases	Deaths under Five Years of Age
Before filtration:							
1880	†90,758						
1885	92.841	1,993	49	182	299	101	685
1886	93.257	‡2,005	‡79	‡255	‡302	‡117	‡667
1887	93,674	2,020	71	203	337	121	561
1888	94,090	2,332	74	283	312	137	773
1889	94,507	2,262	77	297	294	81	741
1890	†94,923	2,265	62	386	327	124	641
1891	94,846	2,383	108	421	310	150	632
1892	94,769	2,558	50	448	298	165	863
1893	94,692	2,140	58	381	257	103	578
1894	94,615	2,162	52	326	301	118	656
1895	94,538	2,345	162	311	287	147	697
1896	94,461	2,099	97	244	298	126	556
1897	94,384	2,013	84	312	259	107	490
1808	94,307	1,904	94	225	238	88	464
1899	94,230	1,991	82	263	248	72	510
After filtration:							
1900	†94,151	1,742	38	256	231	70	382
1901	94,996	1,751	20	220	239	42	298
1902	95,841	1,623	29	227	215	37	251
1903	96,686	1,808	19	242	228	39	321
1904	97,531	1,848	18	258	216	§	281
1905	¶98,374	1,813	19	227	212	§	329
1906	99,219	1,770	20	§	193	§	322
1907	100,064	1,900	20	§	177	§	313

* From Reports of the N.Y. State Dept. of Health.

† U.S. Census. ¶ State Census. Other populations interpolated by the "arithmetical" method.

‡ Eleven months only, December not being given.

§ Title discontinued in reports.

Typhoid fever, which had been excessively prevalent, dropped immediately after the introduction of filtration to a much lower level. In addition to this there took place a decrease in the total death-rate minus the typhoid component, Albany thus furnishing another illustration of the Mills-Reincke phenomenon. This fact was noticed and reported by Mr. Mills and others, before our work began.

The decreases in the mortality from certain diseases at Albany are somewhat different in degree from those observed at Lawrence, Lowell, and Hamburg. Altho the death-rates at Albany from acute respiratory diseases, pulmonary tuberculosis, and "diarrheal diseases" (not including typhoid fever) were lower after the introduction of filtration than before, it must be noted that all three rates had been declining

before that time, and that in none of them does there appear any greater rapidity of decline setting in at this time. The most noticeable result shown by any curve of chart (except that for typhoid fever) appears in the death-rate under five years of age, for which the rate of decline just subsequent to 1899 was somewhat greater than it had been before. Falling to about 300, it continued

TABLE 18.

TOTAL DEATH-RATES, AND DEATH-RATES FROM CERTAIN DISEASES, ALBANY, N.Y., 1885 TO 1907 INCLUSIVE, PER 100,000 OF POPULATION.*

Year	Total Death-Rate	Typhoid Fever	Acute Respiratory Diseases	Pulmonary Tuberculosis	Diarrheal Diseases	Deaths under Five Years of Age
Before filtration:						
1885	2,147	53	196	322	109	738
1886	†2,150	†85	†274	†324	†126	†716
1887	2,157	76	217	360	129	599
1888	2,478	79	301	332	146	821
1889	2,393	81	314	312	86	784
1890	2,386	65	407	345	131	675
1891	2,512	114	444	327	158	667
1892	2,699	53	472	315	174	911
1893	2,260	61	402	271	109	610
1894	2,285	55	345	318	125	694
1895	2,480	171	329	304	156	738
1896	2,222	103	259	316	134	589
1897	2,133	89	331	274	114	519
1898	2,019	100	239	252	93	492
1899	2,113	87	279	263	76	542
After filtration:						
1900	1,850	40	272	246	74	406
1901	1,843	21	232	252	44	314
1902	1,693	30	237	224	39	262
1903	1,870	20	250	236	40	332
1904	1,895	18	265	222	‡	288
1905	1,843	19	231	216	‡	334
1906	1,783	20	‡	194	‡	325
1907	1,899	20	‡	177	‡	313

* Computed from preceding table. Slow sand filter since September, 1899, for Hudson River water, which furnishes about two-thirds of total supply. One-third of supply, both previous and subsequent to introduction of filtration, has been composed of impounded surface water.

† Eleven months only.

‡ Title discontinued in reports.

at that level up through the last year we have taken, 1907. This phenomenon is not, however, clearly reflected in the curve for total death-rate minus typhoid component, which shows no greater rapidity of decline after introduction of filtration. But even the fact that the decline in total death-rate continued as rapid as before may be in part due to the improvement of the water-supply, for it might perhaps otherwise have risen. The effect, nevertheless, is not nearly so clear-cut as in the cases of Hamburg, Lawrence, and Lowell, and requires explanation. Such explanation we believe

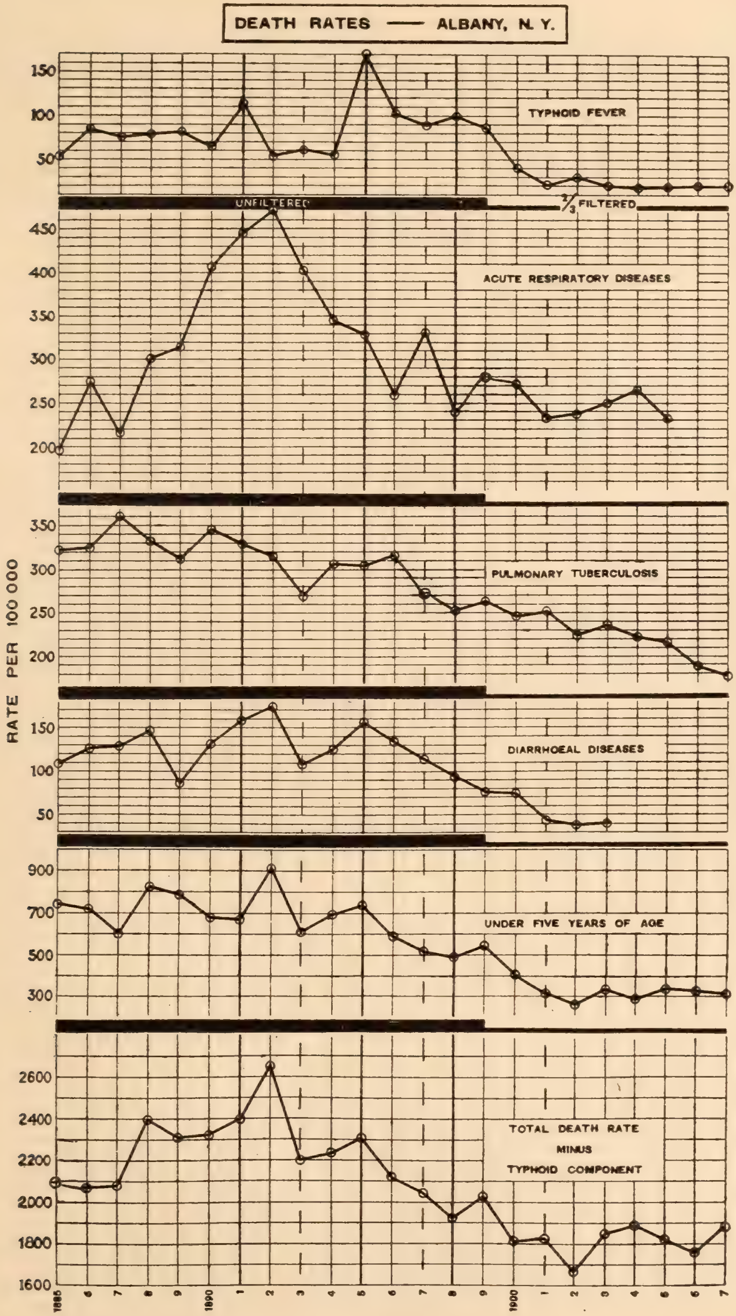
DEATH RATES — ALBANY, N. Y.
TYPHOID FEVER
UNFILTERED
2/3 FILTERED
ACUTE RESPIRATORY DISEASES
PULMONARY TUBERCULOSIS
DIARRHOEAL DISEASES
UNDER FIVE YEARS OF AGE
TOTAL DEATH RATE
MINUS
TYPHOID COMPONENT
RATE PER 100 000

Chart 9.

may possibly be found in the fact that one-third of the water-supply has come, and still comes, without purification, from the surface sources mentioned, the quality of which is said to be somewhat uncertain. It is possible at any rate that if the whole of a supply be not purified, the resultant drop in total death-rate minus typhoid component may be much less, even tho there be, as in this case, a decided diminution in typhoid fever. We greatly regret that we have had no such personal familiarity with conditions at Albany as we have enjoyed with the various cities on the Merrimac River, and that we must leave to others possessing fuller acquaintance with local conditions the task of clearing up local difficulties.

From average death-rates for periods of five years before and after the introduction of filtration (omitting the year 1899 itself), we compute that for every death less from typhoid fever after that date, there were still, even here, 4.1 fewer from other causes. This ratio is of course uncorrected for diminutions in mortality due to other factors than filtration, so that, tho affording an approximate indication, it cannot strictly be substituted in Hazen's theorem.

2. *Binghamton, N.Y.* — Among other examples of the Mills-Reincke phenomenon which have come to our attention, we may include Binghamton, N.Y., altho this city exhibits great irregularities which distinguish it from the examples we have already discussed. Binghamton took its water-supply without purification from the Susquehanna River until June, 1902, when mechanical filtration was introduced. Certain vital statistics for this city are given in Table 19.

From these figures our Chart 10 has been constructed. It will be observed that, following the introduction of filtration, the typhoid fever death-rate, which had been excessive, tho irregular, fell to a low level and remained there. The total death-rate minus typhoid component reflected this fall, altho one year, 1904, shows a rate higher, with the exception of only two previous years, than any rate since at least 1885. This irregularity shows that this is not altogether a clear case of the Mills-Reincke phenomenon, and therefore requires for a complete solution further elucidation.

Altho the authors have been unable to give to the case any local study such as it imperatively demands, certain facts deserve

mention. In the first place, both curves had before filtration shown very great irregularities, that for total death-rates minus typhoid component in particular having at one time fluctuated from 13 to 20 inside of four years. Typhoid fever had in certain years receded to a comparatively low rate, and the same is true of the total death-rate. When we consider, also, the small numbers with which we have to deal and the consequent unsteadiness of the statistics, it becomes evident that, in the absence of more detailed and thorough local studies, too much reliance should not be placed upon them.

TABLE 19.

VITAL STATISTICS OF BINGHAMTON, N.Y.*

YEAR	POPULATION	TYPHOID FEVER		TOTAL DEATHS	TOTAL DEATH-RATE PER 1,000
		Deaths	Death-Rates per 100,000		
Before filtration:					
1880	†17,317				
1886	27,929	3	11	333	11.9
1887	29,698	14	47	433	14.6
1888	31,467	32	102	493	15.7
1889	33,236	34	102	465	14.0
1890	†35,005	21	60	545	15.6
1891	35,469	32	90	668	18.8
1892	35,933	18	50	667	18.6
1893	36,397	16	44	622	17.1
1894	36,861	18	49	584	15.8
1895	37,325	13	35	540	14.5
1896	37,789	9	24	494	13.1
1897	38,253	13	34	498	13.0
1898	38,717	28	72	584	15.1
1899	39,181	10	26	681	17.4
1900	†39,647	17	43	822	20.7
1901	40,125	21	52	755	18.8
1902	40,603	11	27	725	17.9
After:					
1903	41,081	4	10	619	15.1
1904	41,559	4	10	762	18.3
1905	‡42,036	5	12	678	16.1
1906	42,514	5	12	678	15.9
1907	42,992	8	19	673	15.6

* Compiled and computed from Reports of N.Y. State Dept. of Health.
† U.S. Census ‡ State Census. Other population figures interpolated by the "arithmetical" method.
Mechanical filter since June, 1902.

On going through the Annual Reports of the New York State Board of Health in an endeavor to discover, if possible, the specific reason or reasons for the peak in total death-rate minus typhoid component in 1904, we have found that there did indeed occur in that year in Binghamton epidemics of measles and diphtheria and an unusual number of deaths from phthisis. These, however, explain only one-third of the excess of the rate for this year over the average rate

for the following three years. In short, we have been unable to arrive at a satisfactory explanation of the irregularity of the total death-rate curve since 1902. We have nevertheless included Binghamton in

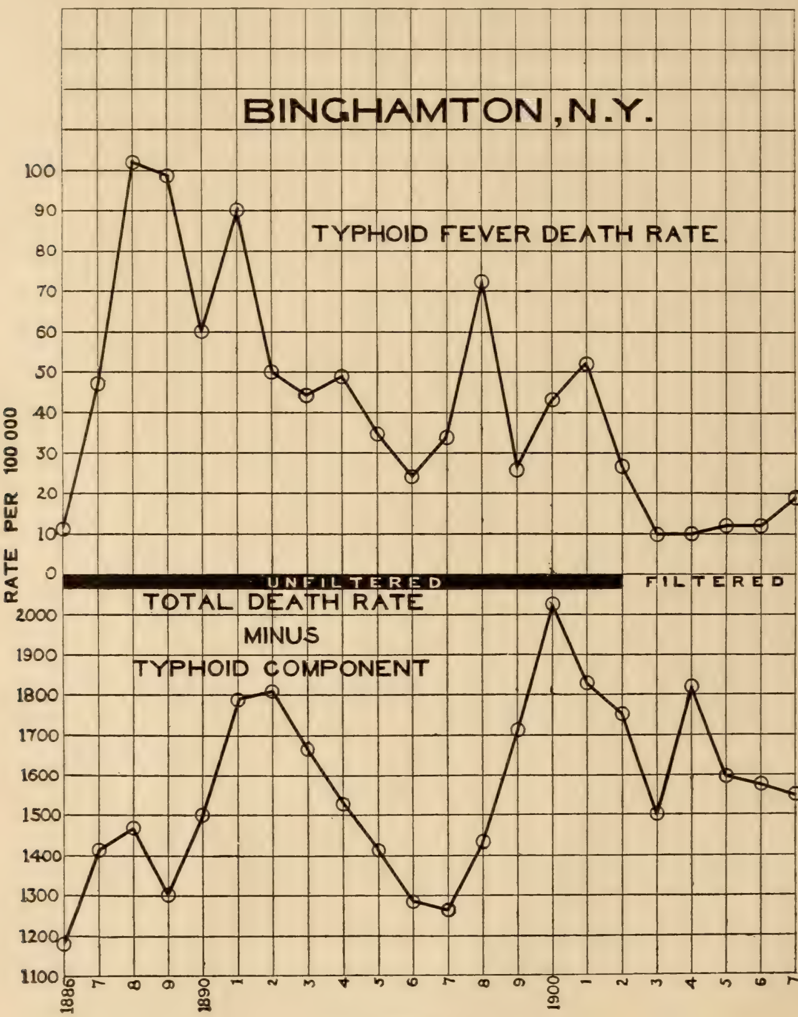

CHART 10.

our studies as an example, tho not a very clear one, of the Mills-Reincke phenomenon.

Taking periods of five years before and after filtration, we compute

that for one death less from typhoid fever afterward there were 1.5 fewer from other causes—this being merely an uncorrected ratio not applicable directly as a test of Hazen's theorem.

3. *Watertown, N.Y.*—As a city which had suffered severely from typhoid fever and which, after a noted epidemic in 1904, had installed a mechanical filter plant, we were led to study *in absentia* Watertown, N.Y. In the foregoing section we have presented an atypical instance of the Mills-Reincke phenomenon, and we now submit a demonstration of a city where that phenomenon did not apparently occur at all after the filtration of the public water-supply.

Until 1904 Watertown took its water from the Black River, a stream polluted by the populations of a number of towns above. In September, 1904, mechanical filtration was introduced immediately following the famous typhoid fever epidemic studied and reported upon by Dr. George A. Soper. A comprehensive description of the city and its past and present water-supply is given in Dr. Soper's paper "The Management of the Typhoid Fever Epidemic at Watertown, N.Y., in 1904."[1]

We present herewith certain vital statistics for this city:

TABLE 20.
VITAL STATISTICS OF WATERTOWN, N.Y.*

YEAR	POPULATION	TYPHOID FEVER		TOTAL DEATHS	TOTAL DEATH-RATE PER 1,000
		Deaths	Death-Rates per 100,000		
Before filtration:					
1880	†10,697				
1888	13,919	7	50	294	21.1
1889	14,322	4	28	224	15.6
1890	14,725	6	41	234	15.9
1891	15,422	6	39	285	18.5
1892	16,119	8	50	371	23.0
1893	16,816	9	54	336	20.0
1894	17,513	15	86	312	17.8
1895	18,210	31	170	331	18.2
1896	18,907	9	48	312	16.5
1897	19,604	12	61	340	17.3
1898	20,301	19	94	291	14.3
1899	20,998	18	86	351	16.7
1900	†21,696	22	101	397	18.3
1901	22,446	8	36	347	15.4
1902	23,196	15	65	317	13.7
1903	23,946	17	71	356	14.9
1904	24,696	52	211	413	16.7
After:					
1905	‡25,447	6	24	384	15.1
1906	26,197	13	50	461	17.6
1907	26,947	10	37	504	18.7

* Compiled and computed from Reports of N.Y. State Dept. of Health.
† U.S. Census. ‡ State Census. Other population figures interpolated by the "arithmetical" method. Mechanical filter since September, 1904.

[1] *Jour. N.E. Water Works Assn.*, 22, p. 87.

For Watertown as for Binghamton we have plotted the general death-rate and that from typhoid fever (Chart 11). The irregularities in both curves are even more evident than in the case of Binghamton. Typhoid fever was apparently not reduced by the introduction of filtration to as low and as permanent a level as should have been expected, for in 1906 we find that the rate had again risen to 50. The total death-rate minus typhoid component, instead of showing a decrease in 1905, 1906, and 1907, actually shows a rapid rise. Here, then, we find apparently the direct reverse of the Mills-Reincke phenomenon, and this fact requires explanation. The authors have been unable to undertake any complete and personal investigation of the local conditions, but may venture to point out briefly several factors possibly of importance.

As in the case of Binghamton, we must regard as suspicious the great irregularities of the curves. Even at certain times of high epidemic prevalence of typhoid fever—notably in 1904, and also in 1895 and 1898—the total death-rate remained at a comparatively low level; while the highest total death-rate shown on the diagram occurred in a year (1892) when typhoid fever, tho excessive, and not reached nearly the proportions of later years. These fluctuations are undoubtedly due in part to the small numbers dealt with, for in a population of only 21,696 (for 1900) the small number of deaths each year leaves much room for serious percentage fluctuations. This circumstance, however, can hardly furnish more than the beginning of an explanation.

A much more important fact in connection with the non-appearance of the Mills-Reincke phenomenon is that the typhoid fever death-rate itself was not reduced after filtration to nearly as low a level as would have been expected. Even after nearly two years from the beginning of filtration, we find a rate of 50 (in 1906), while for 1907 and 1908 (figures for the latter year just published) the rates were 37 and 40 respectively. These rates throw a strong suspicion on the efficiency of purification, and if a considerable amount of infection still remains in the filtered water, the Mills-Reincke phenomenon ought not to have been expected to appear. This point we wish to emphasize particularly, for, if the excess of typhoid fever in Watertown in 1906–8 was due to the water, that fact would

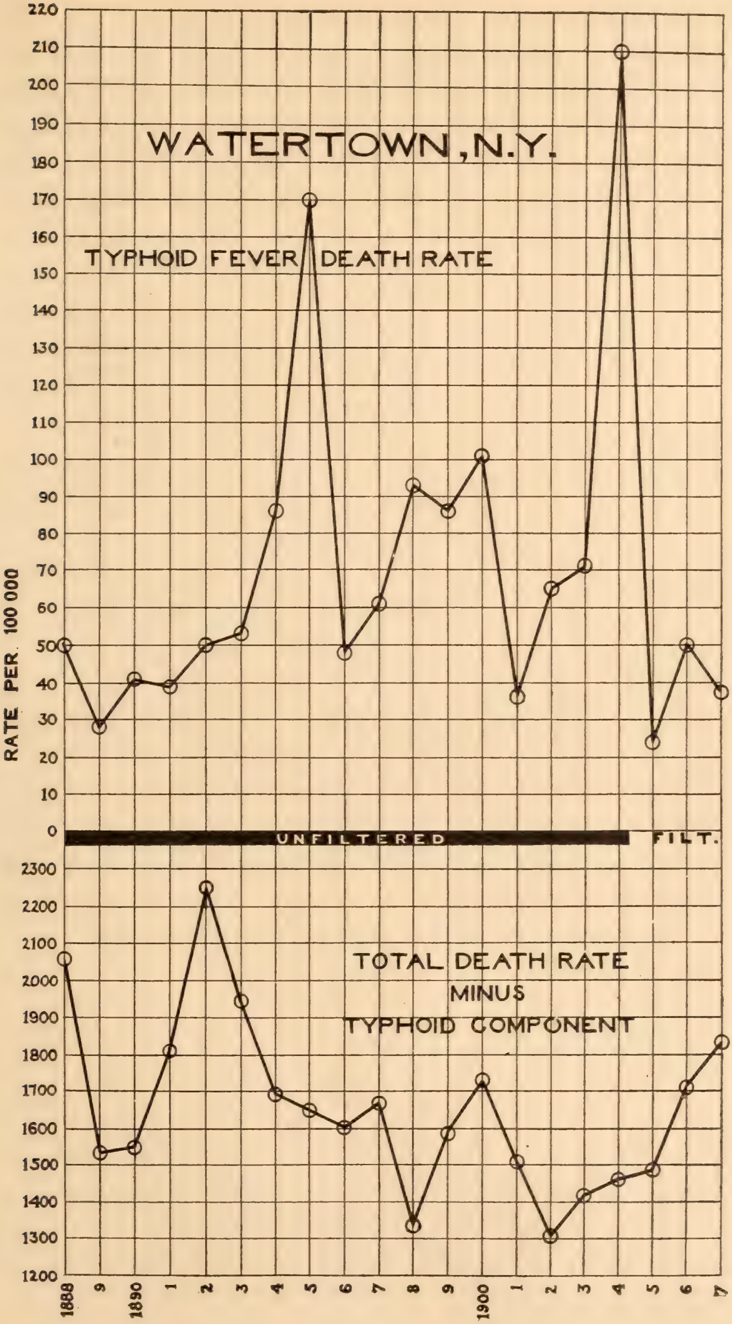
WATERTOWN, N.Y.
TYPHOID FEVER DEATH RATE
RATE PER 100 000
UNFILTERED
FILT.
TOTAL DEATH RATE
MINUS
TYPHOID COMPONENT
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
2300
2200
2100
2000
1900
1800
1700
1600
1500
1400
1300
1200
1888
9
1890
1
2
3
4
5
6
7
8
9
1900
1
2
3
4
5
6
7

CHART II.

afford sufficient reason for the absence of the Mills-Reincke phenomenon.

We understand that acids or other chemical substances in the waters of the Black River have at times, especially of low water, been present in sufficient concentration to interfere with the proper operation of the city filters. Evidence of such chemical pollution is not far to seek.

It is from the pulp and paper industries which have become well established on the river that the present gross pollution of the river is due. A number of mills are situated on the watershed with an aggregate output of over 300 tons of paper per day, in the manufacture of which sulphite pulp is used. Watertown is an active manufacturing city, and the river shows unmistakable signs of a high degree of pollution. All kinds of refuse are thrown directly into the stream, the paper mills and other factories using water in abundance to carry out factory wastes (Monthly Bulletin, N.Y. State Board of Health, July, 1908, p. 190).

The effect of the chemical waste is quite apparent in times of low water in the river. A change of color of 25 parts per million (platinum scale) in four hours is not unknown here. . . . . This color change is frequently accompanied by a decrease in the alkalinity of the raw water (F. H. Jennings in discussion of above-mentioned paper by Dr. Soper, p. 137).

It may be objected that if there were sufficient concentrations of chemical wastes in the river to interfere with the operation of the filters, most or all of the bacteria in the water would be killed by their disinfecting properties, for there is no doubt that such wastes as those from pulp mills have a marked germicidal effect.[1]

But it is probable that the composition of the water fluctuates widely and rapidly, as is asserted in one of the above quotations, and it is quite conceivable that water of high disinfecting powers with low bacterial content today might incapacitate the filters for dealing with water of low disinfecting power and high bacterial content tomorrow.

XVIII. FLUCTUATIONS OF POPULATION IN PERIODS OF PANIC AND PROSPERITY AS SOURCES OF STATISTICAL ERROR.

Very early in our studies we were struck with the fact that the beginnings of filtration in Lawrence and of substitution of groundwater at Lowell coincided almost exactly with the panic of 1893,

[1] See M. O. Leighton, "Prelim. Report on the Pollution of Lake Champlain," *Water Supply Paper No. 121, U.S. Geol. Survey, 1905*, Report of E. B. Phelps, p. 111. Also E. B. Phelps, "Pollution of Streams by Sulphite Pulp Waste," Paper No. 226 of *ibid.*, p. 7. Cf. *Penn. Health Bull.*, November 1909, on "The Germicidal Effect of Water from Coal Mines and Tannery Wheels upon Bacillus Typhosus, Bacillus coli, and Bacillus anthracis."

and the question naturally arose as to whether the diminished death-rates observed might not have been due, wholly or in part, to the influence of the panic. Our attention was first drawn to this important matter by newspaper references touching the effect of hard times upon health, particularly by an editorial in the Springfield (Mass.) "Republican" of June 30, 1908, from which it appeared that hard times are accompanied by a marked improvement in the public health. Corollaries were drawn to the effect that such improvement was doubtless due to less overworking, less overeating, less overdrinking, and the like—in short, to saner and more wholesome methods of living. And, certainly, following the period of financial depression in the United States setting in late in 1907, unusually low death-rates have been reported from many American cities. An article which has come to our notice, "Era of Low Mortality Reached by the Civilized World,"[1] states, for example, that for 1908 the death-rate for the registration states was, according to the U.S. Census, only 15.3 per 1,000 of population, this being, according to Dr. C. L. Wilbur of the Census, "probably the lowest death-rate that has ever occurred in the United States."

Our attention was independently drawn to the possibility of such an explanation as above mentioned of the lower death-rates at Lawrence by an employee connected with the registration of vital statistics in that city, who stated that to his personal knowledge there had taken place there, at the time of the panic of 1893, a marked change in the habits of the people, who began to spend more time in the open air, to make excursions into the country, and in general to live in a more leisurely and healthful manner. The new mode of life thus imposed upon the people was, it was argued, continued by force of habit even after the mills had again resumed full operation, more vacation time being then demanded by the employees. In the same discussion it was asserted also that there was a considerable change in the character of immigration at this time. It is, in fact, common knowledge among persons familiar with conditions in industrial cities that such phenomena do take place to a greater or less extent in hard times, with reverse effects in times of prosperity; and lower death-rates certainly have appeared in some cities at times of industrial depression.

[1] *Boston Med. and Surg. Jour.*, November 4, 1909.

On the other hand, there is good reason for believing that the simplest and often the chief explanation of the diminutions in the *computed death-rates* will be found in a temporary decline in industrial urban populations. In such case estimated populations for intercensal years would be inflated over their true values, and the rates based upon them would give false evidence of improvement in the public health. Thus there may be in many cases a considerable error which would be made manifest if annual, instead of quinquennial, or decennial, censuses were taken. This would of course be true of the U.S. Census method of estimation (used by us throughout), which gives for such cities, regardless of such possibilities, the same absolute increment from year to year between any two censuses.

To any objection based on a supposed effect of the panic of 1893 on the death-rates of Lawrence and Lowell—either through actual betterment of the public health or through diminished population—we answer first of all by calling to mind Manchester, N.H., a city in all important respects similar, which we have taken as a norm. Some influence upon the death-rates of Manchester, as of other cities, during the hard times might have been expected, but the fact is that no such influence is discernible in the figures. This indicates that the rates for Lawrence and Lowell remained uninfluenced from that source.

Altho satisfied that the results of our studies on Lawrence and Lowell are exonerated by the Manchester data from any serious blame on either of these points, we have thought desirable, nevertheless, to bring forward certain explanatory and confirmatory evidence, particularly in regard to possible diminutions of population in periods of panic. And in doing so we shall include also the case of Hamburg, where a panic was caused by the Asiatic cholera epidemic of 1892.

The cholera panic at Hamburg resulted in a temporary check to the growth of the population such as might be expected from an industrial panic. In fact, not only was the growth checked, but for a time the population was actually diminished, as observed by Dr. Reincke:

> While the population of Hamburg has in recent decades increased regularly by at least 2.6 per cent per annum . . . . it has in 1892 not only failed to increase but has actually decreased by 2,808 persons. . . . . Immigration into the city, which already

at the beginning of the year was small, ceased entirely after the outbreak of the cholera, while emigration increased correspondingly ("Bericht des Medizinal-Inspektorats für das Jahr 1892," Hamburg, p. 1).

The observation thus recorded by Dr. Reincke would have been impossible without accurate statistics for the population from year to year, and we find that such statistics were in fact afforded by annual enumerations of population.

In those years in which no censuses have taken place the Statistical Bureau of the Tax Commission has made since 1868 in the city and suburbs . . . . inquiries on population and dwelling conditions, including all data necessary for official purposes ("Statistisches Handbuch für den Hamburgischen Staat," 4te Ausgabe, Hamburg, 1891, p. 24).

This inquiry, made December 1, commonly called *Umschreibung*, approximates ever more and more the exactitude of the more detailed quinquennial census. Data are taken as to name, age, occupation, etc. The results are not, however, usually worked up in much detail.[1]

We call special attention to the fact that the death-rates given in Dr. Reincke's Annual Reports as well as in the "Sanitary History of Hamburg" (from both of which works we have in this paper freely quoted and drawn data) are based upon these annual enumerations of population.

Without further comment we present the results of the annual enumerations in the following diagram, based on figures taken from the "Sanitary History of Hamburg," p. 28. The dotted lines indicate for comparison the error which would have been introduced had there been only quinquennial or decennial censuses, with interpolation of the other years by the "arithmetical" or U.S. Census method. The maximum error due to this method would in this case have been with the quinquennial only 2 per cent, with the decennial 4 per cent.

It is evident that the recovery of population set in soon again after the panic of 1892–93, and was very rapid.

Turning next to Lawrence and Lowell and the question of the effect of the industrial panic of 1893 in diminishing the population growth of those cities, we have evidence that in them, as in Hamburg after the cholera scare of 1892, recovery in general public confidence

[1] Cf. Reincke, *Gesundheitsverhältnisse Hamburgs im 19. Jahrhundert*, p. 312.

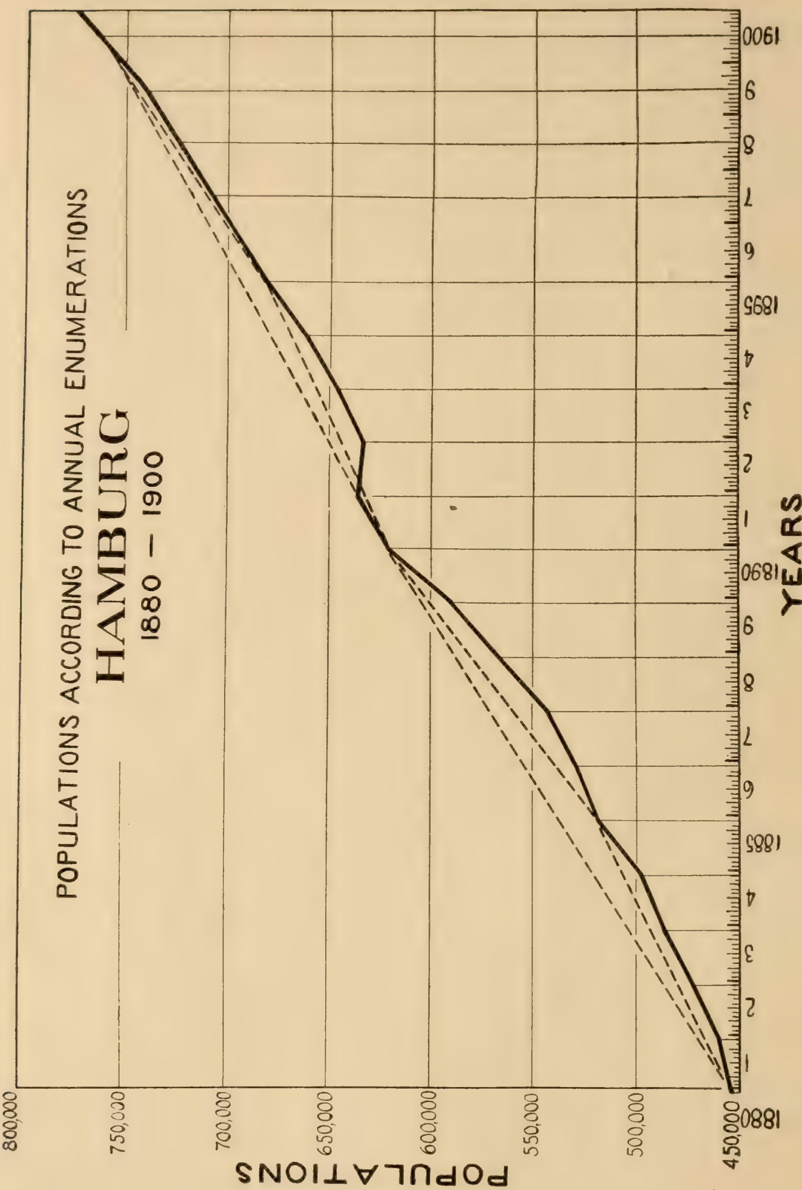
POPULATIONS ACCORDING TO ANNUAL ENUMERATIONS
HAMBURG
1880 — 1900
YEARS
1880
1885
1890
1895
1900
POPULATIONS
450,000
500,000
550,000
600,000
650,000
700,000
750,000
800,000

CHART 12.

and prosperity (with their corollary, increase of population) was early and rapid. There is first of all indication of this in the general financial and industrial movements in the United States at this time.

Past experience has produced a number of instances where, in the second year after a great financial panic, business recovery went ahead so rapidly that it was found necessary, in the next year, to slow up. Of this the classic case in point was 1895. . . . . Sudden revival; active buying of merchandise; prediction of another "boom time"; spectacular rise in stocks, with Europe's capital enlisted; iron production surpassing all monthly records—these things were witnessed in the second year after 1893 ("The Nation," "Finance," February 24, 1910).

And this description is confirmed by the populations for Lawrence and Lowell given by the State Census of 1895. The characters of the populations had also, according to this census, remained practically the same.[1] Thus any fluctuations in quantity or character of population which may have taken place in 1893–94 were chiefly temporary. And certainly the much diminished death-rate computed for 1895 cannot be ascribed in any significant degree to error in the population statistics employed.

As an approximate indication of year to year fluctuations of population in Lawrence and Lowell, we have obtained, by courtesy of their city assessors, the total number of polls in those cities (enumerated for purposes of taxation), including practically all males over 20 years of age, as of May 1 of each year since 1889. These figures we have plotted in the following diagram, together with the total populations estimated by the so-called arithmetical method.

The panic and hard times of 1893–94 and the industrial depressions of 1903–4 and 1907–8 are seen to be clearly reflected in the curves for polls. The extent of fluctuation in these cities about 1894 was apparently somewhat greater than at Hamburg in 1892 and the following years. The indication from the Census of 1895 that the check to increase of population in 1893–94 was merely temporary, is confirmed. There was apparently also a check to growth of population in both cities following 1896, and this may perhaps be explained as a result of the secondary industrial depression which followed the revival of 1895.

The ratio of polls to total population remained fairly constant in both cities for the four census years, though more nearly so for

[1] Cf. sec. xv.

Lawrence than for Lowell. Thus for Lawrence in 1890 there were 3.88 persons in total population to each poll; in 1895, 3.69; in 1900, 3.82; and in 1905, 3.84. For Lowell the corresponding figures are

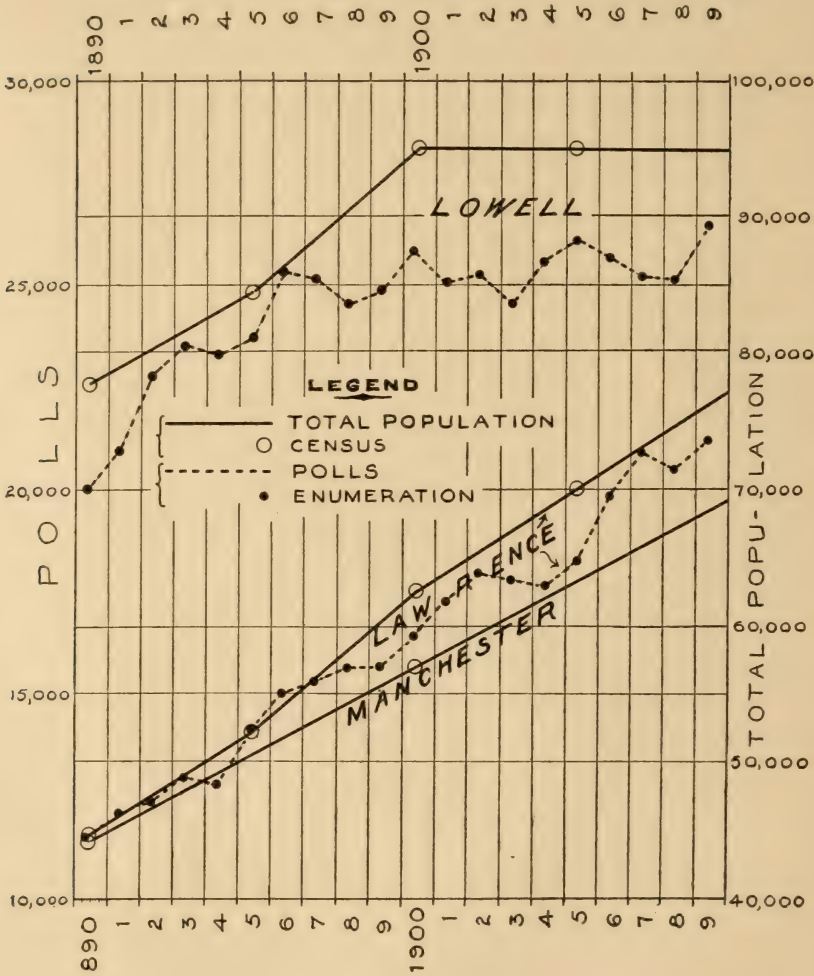

CHART 13.

3.88, 3.56, 3.68, and 3.64. These ratios afford a rough check on the accuracy of the poll enumerations, which tho of uncertain reliability, appear to be sufficiently accurate for the present purpose—i.e., an approximate indication of fluctuations of population.

In 1905 there was a diminution of the population of Lowell not paralleled at Lawrence, a difference which we have left without investigation as being unessential to our study.

We finally conclude, as to fluctuations in magnitude and character of population as sources of statistical uncertainty, that in particular years, especially where only a decennial census is to be had, errors seriously impairing the accuracy of death-rates computed from estimated populations may be introduced. The indications, however, from the curves just given, that the populations of Lawrence and Lowell had in 1895 recovered much of their former growth energy in spite of year to year fluctuations, taken with the evidence given in sec. xv on the elements of the populations and in secs. xv and xvi on Manchester as a norm, lead to the conclusion that no error seriously affecting our results can be attributed to fluctuations in magnitude and character of population.

XIX. SUMMARY AND CONCLUSIONS.

We may now bring together the various results of our study, as follows:

There is abundant evidence of the occurrence of the Mills-Reincke phenomenon, not only in the vital statistics of the cities of Lawrence and Hamburg, studied by Messrs. Mills and Reincke, respectively, but also for Lowell, Albany, and Binghamton. The phenomenon seems at first sight to be wanting in Watertown; but it is possible that in Watertown the purification of the public water-supply has been as yet relatively imperfect, and it is interesting to note that under these circumstances no diminution of the general death-rate has as yet occurred. For a fuller discussion of this point we must refer to the body of the paper.

We have not extended our studies over a larger range of cities, partly for the reason that trustworthy statistics for American cities are by no means common, and partly because of the incomplete and unsatisfactory character of investigations carried on at a distance. We earnestly hope that students of sanitary science who may fortunately have access to the necessary data for other cities will not fail to study and report upon the problems here raised. It is also greatly to be desired that similar studies should be made on the data afforded

by such cities as Washington, D.C., in which the filtration of the public water-supply has not been followed by any material decline in the death-rate from typhoid fever; for in this case such filtration would not be expected to have produced any material decline in the total death-rate. Whether or not a partial purification of a public water-supply may be expected to be followed by a moderate decline in the total death-rate we cannot say, but the experience of Watertown would seem to indicate that this is not necessarily the case.

Mr. Hazen's quantitative expression for the Mills-Reincke phenomenon, when applied to the cities which we have studied (with the exception of Watertown), appears sound and conservative. It seems likely, however, that it will be impossible in the future to confine the relation even within the broad numerical limits suggested by Mr. Hazen. In fact, Mr. Hazen himself is very careful in this particular, as will be seen by a reference to his original statement (p. 510). It is probable that the pollution of a public water-supply may consist, at one time or in one place, of much typhoidal infection mingled with comparatively little sewage, or on the other had, of much sewage only lightly charged with typhoid fever germs. In the former case the reduction in typhoid fever might be out of all proportion to the reduction in general death-rate, and in the latter case vice versa. For Hamburg we have pointed out above that the saving in typhoid mortality was slight in comparison with the saving of mortality in other diseases combined, i.e., roughly only *ca.* 1 to 16. In the other cities studied we find ratios widely different from this, e.g., at Lawrence, 1 to 4.4, at Lowell 1 to 6.0, in Albany, *ca.* 1 to 4.1 (uncorrected), and in Binghamton only *ca.* 1 to 1.5 (uncorrected). It is clear, therefore, that Hazen's theorem is merely a convenient formula rather than a precise mathematical expression.

One of the most surprising results of our study will probably be the disclosure of the remarkable relation subsisting between polluted water and infant mortality. This subject has been more fully eluci dated by Dr. Reincke than by anyone else, as will be evident from a perusal of his discussions quoted above. Our graphical demonstrations, however, are worthy of notice, as they bring out strikingly the principal facts. Students of preventive medicine will do

well to extend these studies, which promise to shed much light upon the solution of one of the most serious problems of the time.

Closely associated with infant mortality stand diarrhea and gastro-intestinal disorders (90 per cent of which in Hamburg occurred among infants under one year of age) in relation to polluted water, which now bids fair to assume a causal importance in these diseases second only to that of contaminated milk.

In regard to tuberculosis the evidence, tho less striking, is interesting and suggestive. Inasmuch as we have been unable, even after the most careful investigation, to discover any other possible explanation of the figures, we are forced to the conclusion that a considerable portion of the decline in mortality from tuberculosis in Lawrence and Lowell during the years immediately following a change from polluted water-supplies was due to that change; and in line with this conclusion a similar explanation appears more than probable for Hamburg.[1]

For pneumonia, bronchitis, and the remaining infections, our data and diagrams speak with clearness, and we need not repeat the discussions we have already given in the preceding sections.

We have examined in detail the influence of fluctuations of population upon our statistical results, and find that no serious error has been encountered by us in that direction. It is certainly an interesting fact that the great panics have rarely if ever coincided with either federal or state censuses; but we find no reason to believe that the declines in either typhoid fever or the general death-rate in Hamburg, Lawrence, or Lowell, altho these happened to coincide with periods of panic, were in fact attributable to decrease of population.

Finally, the question naturally arises, to what is the decline of mortality observed in the Mills-Reincke phenomenon for diseases other than typhoid fever due? A little reflection will show that increase of vital resistance, due perhaps to the use of a purer drinking water, might produce this effect, while, on the other hand, the same results might be reached by an exclusion of disease germs formerly present and working upon the bodies of their victims. Or, as a

[1] See p. 185 of our paper in the volume *Tuberculosis in Massachusetts*, mentioned on p. 491 above.

third possibility, the phenomenon might be due to a combination and co-operation of these two factors.

It is interesting to observe that Mr. Mills in his writings upon this subject, without especially committing himself to either hypothesis, has apparently had in mind chiefly an increase of vital resistance, while Dr. Reincke has expressed himself rather as looking to the removal of disease germs previously present.

The facts at present in our possession do not allow us to settle the question beyond peradventure, and this problem, like many others raised throughout our paper, requires further elucidation.

THE PHYSIOLOGY OF ANAPHYLACTIC "SHOCK" IN THE DOG.*†

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THE investigation here presented was incidental to a study of the treatment of various experimental conditions of low blood pressure,[1] one of which was that associated with anaphylaxis in the dog. As a knowledge of the mechanism of the various forms of low pressure was essential to treatment, extensive physiological studies were made, and as the results of the study of anaphylactic shock are striking and have a very definite bearing on the problems of anaphylaxis in general, they are, therefore, made the subject of this communication.

Physiological and pharmacological studies of anaphylaxis are few in number and with the exception of the work of Arthus on the rabbit and Biedl and Kraus on the dog are limited to the guinea-pig. The general opinion[2] has been that the underlying cause is a change in the cells of the central nervous system and this view has had some support from the observations of Besredka, who found that the clinical manifestations of anaphylaxis could be lessened by ether anesthesia, and of Banzhaf and Famulener, who found a hypnotic dose of chloral hydrate would prevent symptoms in sensitized animals. The observations of these writers are, however, somewhat contradictory, and Rosenau and Anderson state that altho ether anesthesia may mask the symptoms, it does not prevent death.

Attempts to demonstrate specific anatomical lesions have been most unsatisfactory. Gay and Southard state that "we have to

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[1] R. M. Pearce and A. B. Eisenbrey, "A Study of Experimental Conditions of Low Blood Pressure of Non-Traumatic Origin," *Archives of Internal Medicine*, 1910 (in press).

[2] For the general literature of anaphylaxis, see the recent excellent summary by J. F. Anderson and M. J. Rosenau, *Archives of Internal Medicine*, 1909, 3, p. 519.

deal with an intimate cell reaction, demonstrable by definite cell lesions." They describe hemorrhages in the gastric mucosa and fatty changes in voluntary muscle, heart muscle, and nerve fibres. These observations have been confirmed in detail by Tscharnotzky.[1] The respiratory disturbance has been especially studied, first by Gay and Southard and lately by Auer and Lewis. The former consider the cause of death to be respiratory; respiration ceases in the inspiratory phase with gross and microscopic evidence of emphysema. A striking feature is severe diaphragmatic spasms. Rosenau and Anderson state, in view of the persistence of the heart beat after failure of respiration, that death is due to an effect on the nervous control of respiration. Auer and Lewis[2] conclude that anaphylactic death is due to asphyxia produced apparently by a tetanic contraction of the smooth muscle of the bronchioles. This contraction gradually occludes the lumen of the bronchioles and finally no air enters or leaves the lung, and in spite of evident respiratory efforts, the animal is strangulated. The lungs are distended in an inspiratory position and do not collapse when the chest is opened. The condition is due to a peripheral action, for destruction of spinal cord and medulla in no way affects the occurrence of the condition of the lung. Auer and Lewis do not care to give an opinion as to whether the toxic dose exerts its effect on the bronchial muscles alone or upon the vagus motor endings, or both. They also describe an initial rise in blood pressure, with later a gradual drop to 10 to 20 mm. of mercury or less, within 10 minutes. Heart block, probably due to asphyxia, also develops. Auer and Lewis also make the interesting observation that the administration of atropin, which paralyzes the bronchial muscles, causes a relaxation of the rigid anaphylactic lung, and that if atropin is given before the toxic dose of horse serum the pulmonary symptoms may be greatly reduced or prevented entirely. Anderson and Schultz[3] confirm most of these observations, but find that the administration of chloral hydrate, urethrane, adrenalin, and pure oxygen also may prevent the death from asphyxia. When life is thus prolonged, death results from the condition of low blood pressure.

[1] "Travail de l'Institut Bact. de Moscow, 1909," *Ref. Bull. de l'Institut Pasteur*, 1909, 7, p. 591.

[2] *Jour. Amer. Med. Assoc.*, 1909. 53, p. 458; also *Jour. Exper. Med.*, 1910, 12, p. 151.

[3] *Proc. Soc. Exper. Biol. and Med.*, 1909, 7, p. 32.

Arthus,[1] who has worked with the rabbit, describes a very striking fall in blood pressure with polypnea, involuntary evacuation of feces and suppression of urine.

Anaphylaxis in the dog has not been extensively studied. This is possibly due to the negative results of Remlinger, and in part also to the fact that the phenomena in the dog differ in some respects from those in the guinea-pig. The chief differences are (1) the absence in the dog of the respiratory disturbance so characteristic in the guinea-pig and (2) the occurrence in the former animal of a fall in blood pressure which is more striking than that in the guinea-pig. The fall in blood pressure in the dog is considered by Biedl and Kraus[2] who first described it, to be the essential phenomenon of anaphylactic shock and to be due to a primary peripheral vaso-dilatation. These conclusions we have confirmed in an earlier communication.[3]

That the phenomena following an injection of horse serum into dogs previously sensitized with the same serum are to be considered as manifestations of anaphylaxis is shown by the following observations:

1. The intravenous injection of horse serum into a normal dog produces no clinical symptoms and no change in blood pressure.

2. An intravenous injection into a conscious animal three weeks after a subcutaneous injection of the same serum produces the following clinical manifestations:

Dog 50, weighing 4,680 gm., received 5 c.c. of normal horse serum subcutaneously on February 26. Twenty-three days later (March 21) 5 c.c. of the same serum were injected slowly into a superficial vein of the left hind leg under local anesthesia. Before the entire amount was injected, the animal became restless, and made retching movements. This was followed within two minutes by vomiting and involuntary evacuation of feces and urine. During this period, the animal when placed on the floor took a few steps, slowly and with a peculiar stiff-legged gait. At the end of three minutes it fell to the floor slowly and lay on its side with head prone. Retching movements continued. Respiration was somewhat deep and labored and 28 per minute as compared with 36 per minute before injection, no dyspnea; the pulse in the femoral artery could not be felt; superficial reflexes normal. Vomiting and involuntary defecation and micturition continued more or less intermittently for seven minutes after injection. The animal when placed upright could not stand, but this appeared to be due to muscular weakness rather than to paralysis; was indifferent to surroundings but entirely conscious. At the end of 22 minutes the pulse was palpable in the femoral; the animal could stand

[1] *Arch. Internat. d. Phys.*, 1909, 7, p. 471.

[2] *Wiener klin. Wchnschr.*, 1909, 22, p. 363.

[3] R. M. Pearce and A. B. Eisenbrey, *Proc. Soc. Exper. Biol. and Med.*, 1909, 7, p. 30.

alone but preferred to lie down; though 10 minutes later it got up an walked around but was still weak. This degree of recovery occurred about half an hour after injection, and after the lapse of an hour the respirations were 28 per minute, the pulse readily palpable, the animal was weak but had power of locomotion, slight diarrhea was present, and the dog refused food. On the following morning he was found dead, a period of less than 15 hours having elapsed since injection. The cage showed evidence of profuse bloody diarrhea.

Autopsy: No excess of fluid was found in the peritoneal and pleural cavities, the serous surfaces were smooth. The bladder contained a small amount of clear yellow urine. The intestines appeared somewhat dark in color and the lower 15 cm. of the rectum showed numerous pin head petechial spots beneath the peritoneum; larger ecchymotic areas occurred beneath the serosa of the gall bladder. Spleen, liver, and kidneys appeared normal. In the greater curvature of the stomach the mucosa over two areas of about 1.5 cm. in diameter was intensely hemorrhagic and swollen and some superficial erosion of the epithelium was evident. In the region of the pylorus both the stomach and duodenum were normal, but about 2 cm. below the pylorus and for a distance of 27 cm. along the small intestine, the mucosa was greatly swollen and hemorrhagic. Below this area the Peyer's patches were elevated and dark colored, but hemorrhages were absent. A few centimeters below the ileocaecal valve the colon was intensely hemorrhagic with considerable erosion of epithelium. The mucosa of the gall bladder was smooth and showed no hemorrhage. Heart and lungs appeared normal; the latter collapsed on opening the thorax and presented some hypostatic congestion in dependent parts. The brain showed no macroscopic lesions.

3. In animals under complete ether anesthesia the objective manifestations of anaphylactic shock are absent;[1] in all such, however, an immediate and prolonged fall in blood pressure which persists for a considerable time occurs. This has been seen in 40 of 42 animals; in two dogs with pneumonia (distemper) it failed to occur. The lowered pressure is unaccompanied by noteworthy respiratory or cardiac disturbance.

4. After anaphylactic shock has been produced in a sensitized animal and the animal has recovered from the acute manifestations, subsequent injections fail to cause a reaction evident either by clinical symptoms or by change in the blood pressure.

These results, which are in accord with those of Biedl and Kraus, indicate that the principles underlying anaphylaxis in the dog are the same as those for anaphylaxis in the guinea-pig.

We have regarded the fall in blood pressure as the constant and most characteristic phenomenon of anaphylactic shock in the dog, and have based our conclusions concerning the physiology of anaphylaxis on its presence or absence under various experimental conditions.

[1] Vomiting, involuntary defecation and passage of urine, have been observed once in an animal anesthetized by Gréhant's method.

The first experiments had for their object the determination as to whether the condition of low blood pressure was due to changes in the blood caused by the injection of the toxic dose or to changes in the fixt cells. It has been generally assumed in all recent investigations with the guinea-pig that the latter explanation is the correct one, but as far as we are aware this has never been determined by transfusion experiments such as we employed. Furthermore, Biedl and Kraus show that not only is there a change in the numerical relation of the two types of cells of the blood one to another, but also, and this we have confirmed, that a decreased coagubility of the blood occurs. It seemed important, therefore, to rule out at once the influence of possible primary or secondary toxic bodies formed in the circulating blood by the union of the bodies in the toxic dose of serum with those bodies which might be formed in the blood of the animal by the first injection.

Our procedure has been to exsanguinate, under ether anesthesia, a small normal dog (A) and to transfuse this animal by Crile's method with the blood of a larger sensitized dog (B) until the blood pressure reached approximately its original level. After sufficient blood has been obtained from B to raise the pressure of A, the sensitized dog is then bled to exsanguination and transfused from a third normal dog (C) until its pressure reaches its previous normal level. At the proper moment, the normal dog containing the blood of the sensitized dog, and the latter containing the blood of the normal dog, each receives intravenously the toxic dose of horse serum. In the former, a fall in pressure does not occur and in the latter it does, thus proving that the phenomenon of anaphylaxis is due to a reaction in the fixt cells and not either primarily or secondarily to changes in the blood. This result is in accord with the contention of Gay and Southard and of Friedberger that the reaction of intoxication in the guinea-pig is located within the body cells and is opposed to the theory of "antibodies."[1]

The following protocol, presented briefly, is illustrative:

Dog A, weight 6,260 gm., ether anesthesia, cannula in femoral artery, connected with mercury manometer and recording on a kymograph. Original pressure 92 mm. Bled from the carotid artery until no more blood flowed, the pressure dropping to

[1] Compare with views of Friedberger, *Ztschr. f. Immunitätsforsch. u. exper. Therap.*, 1909, 2, p. 208; 1909, 3, p. 581; 1910, 4, p. 612, and Friedemann, *ibid.*, 1909, 2, p. 591.

16 mm. Hg. Transfused from B for 17 minutes by carotid anastomosis, until pressure reached original level, when 5 c.c. of normal horse serum were injected into the femoral vein. No change in pressure except slight mechanical rise.

Dog B (No. 17), weight 8,020 gm. Had received 5 c.c. horse serum subcutaneously 25 days before. Ether anesthesia. Arrangement of cannula as in A. Original blood pressure 90 mm. After transfusion sufficient to raise pressure of A, animal B was bled for 16 minutes until flow ceased, when blood pressure was at a level of 14 mm. Transfusion from dog C was then begun and continued for 24 minutes when permanent level equal to previous normal level was obtained. At this time 5 c.c. horse serum were injected into the femoral vein with an immediate fall in pressure to 24 mm Hg.

Dog C, normal dog, weighing 9,100 gm., prepared as were A and B, and used for transfusion of B.

Having thus determined that the reaction was evidently due to an effect on the fixt cells of the body and presumably on those of the vasomotor system, the question of the general distribution of the blood in anaphylactic shock was studied as a preliminary to the study of peripheral and central influence. For this purpose, experiments were made under the following conditions:

All animals were under full ether anesthesia. The blood pressure was taken from the left femoral artery with a mercury manometer. Injections were made into the right saphenous vein. The changes in blood distribution were determined by oncometric studies by means of guttapercha capsules applied to spleen, kidney, and intestine, by a cannula introduced into the right common iliac vein and projecting into the inferior vena cava, by plethysmographic study of a fore-limb and by a metal cylinder penetrating into the cranial cavity. Each of these was connected by rubber tubing with a bellows recorder allowing simultaneous records on a revolving drum. It was thus possible in a single experiment to obtain simultaneously, as was frequently done, records of volumetric changes in the femoral artery, iliac vein, kidney, spleen, and intestine, with also a record of the respiration, or any combination of these with a record of variation in a limb or in intracranial pressure.

The temperature of the animal was maintained by the use of a hotwater coil encircling the greater part of the trunk and a record of changes in temperature was obtained by a thermometer in the rectum. Normal horse serum was used in doses of 5 c.c., subcutaneously for sensitization, and the physiological experiment was made after about 21 days. The dose used to bring about anaphylac-

tic "shock," usually 5 c.c., has varied from 2 to 6 c.c., altho it must be admitted that no attempt has been made to determine the minimal toxic dose. It has always been given intravenously.

The study of blood distribution included six observations. The fall in arterial pressure, as well as the change in the volume of the kidney, and the character of the respiration, are shown in Fig. 1.

It is apparent that anaphylactic shock is characterized by an abrupt fall of blood pressure averaging 50 to 70 mm. Hg which is independent of initial change in heart action, tho during the continuance of low pressure the amplitude of the pulse wave is greatly diminished, the result presumably of the small amount of blood reaching the heart. Respiratory disturbances, except in as far as they occur as a result of medullary anemia due to the low general arterial pressure, are absent. Oncometric studies show a decrease in the volume of kidney, intestine, and spleen simultaneously with the decrease in arterial pressure. Of these three organs the decrease is, as a rule, most marked in the kidney and least in the intestine. A very slight initial decrease in brain volume has been found as well as a slight diminution in the volume of an extremity. This decreased peripheral circulation is accompanied by an accumulation of blood in the liver and large veins of the abdomen. This has been determined by inspection of the liver and large veins and by the observation that a cannula introduced into the inferior vena cava and connected with a water manometer shows a moderate increase of pressure, equal to 6 to 10 mm. water, at the time of the fall in arterial pressure, with an almost immediate return to normal level as the blood becomes evenly distributed throughout the large venous trunks.

It is evident, therefore, that the important feature of the condition of low pressure here described is a lack of tone of the vessels, particularly of the splanchnic area, characterized by extreme venous congestion from which the animal does not quickly recover. It is essentially the condition characterized as a "bleeding into the veins of the abdomen" and in many respects is analogous to the circulatory disturbance of surgical shock.

The next step, naturally, was to determine to what extent this condition was due to peripheral and to what extent to central vasomoter disturbances. To determine the influence of the central

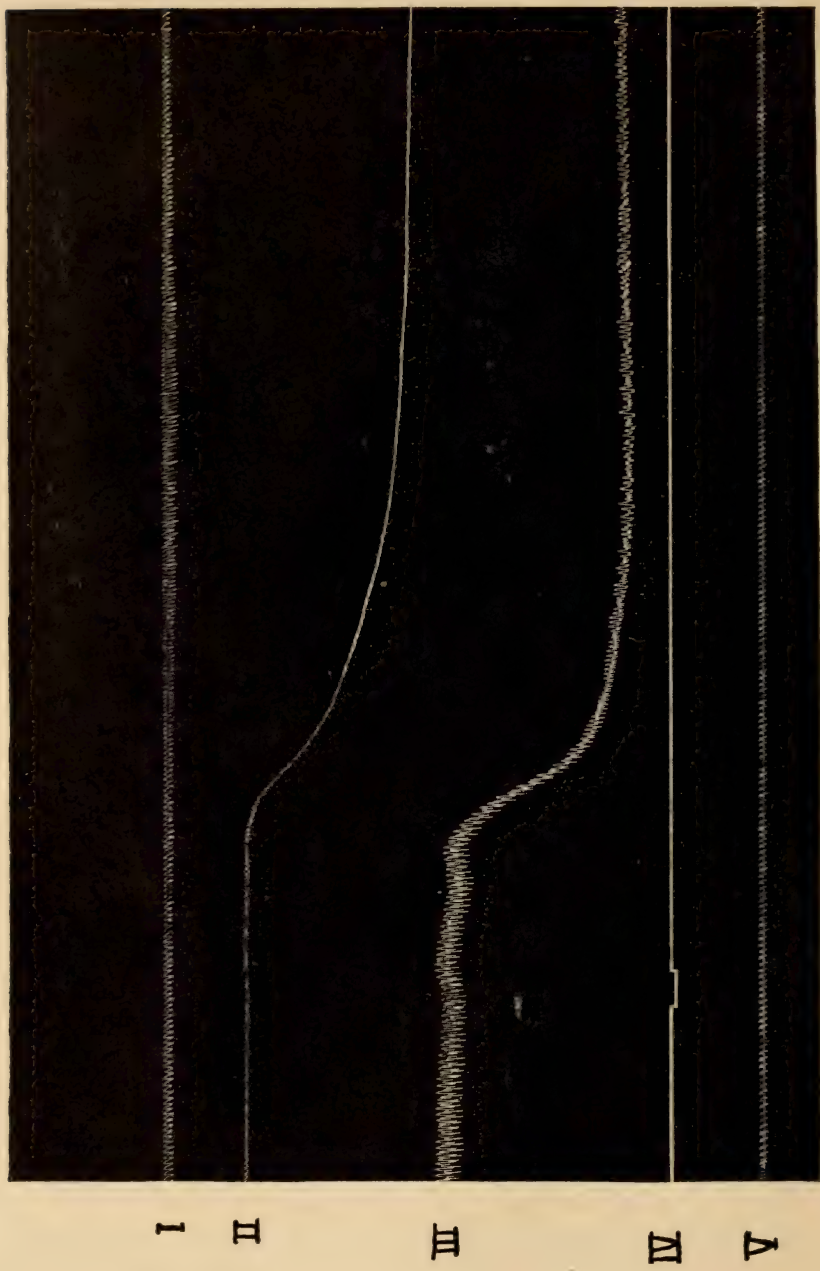

FIG. 1.—This tracing illustrates the effects of the injection of 5 c.c. normal horse serum into the saphenous vein of a dog (weight 8380 grams, that had been given 5 c.c. of the same serum 22 days before. Ether anesthesia. I=respiration; II=kidney volume (oncometer on left kidney); III=blood pressure; IV=base line with signal at time of injection; V=time in seconds.

mechanism, experiments were done under ether anesthesia, in which the spinal cord, vagi, cervical sympathetic and splanchnic nerves were severed. None of these procedures, singly or collectively, prevented the fall in pressure, thus pointing to the absence or slight importance of central influence. Such evidence, tho usually considered conclusive by physiologists, we preferred not to accept without absolutely excluding the influence of centers in the cord as well as the secondary effect of cerebro-medullary anemia. Three groups of experiments were therefore performed. These included the study of anaphylaxis under the following conditions: (1) decapitation, (2) decapitation and destruction of the cord with blood pressure reinforced by transfusion, and (3) with the cerebral circulation independent of the circulation of the body. All experiments of these three groups were done under complete ether anesthesia by tracheal cannula.

1. The object of decapitation[1] was naturally to eliminate all cerebro-medullary influence. After this procedure, it was found that the blood pressure falls to a point about one-half of its original level and remains there with little variation, if care is exercised to keep the artificial respiration uniform and to maintain the body heat, for a period covering the usual duration of the experiments. With the loss of vagus influence the heart rate is quickened and the excursions of the pulse wave shortened. The respiratory curve in the blood pressure tracing is well marked. With these conditions prevailing the toxic dose of horse serum caused a fall of blood pressure to the level occuring from similar doses in intact animals. The onset of the fall was less abrupt and the low level was reached more slowly. That the fall could occur, however, independently of cerebro-medullary influence was clearly shown.

2. In the experiments in which the cord was mechanically destroyed after decapitation, the object was to eliminate all except local vasomotor influences. After destruction of the cord it was found that a further dilatation and fall in blood pressure occurs beyond that caused by the decapitation and that death rapidly ensues. This is apparently due to the fact that with the additional loss of the spinal con-

[1] Decapitation may be performed with a comparatively negligible amount of hemorrhage and therefore with but slight effect on the circulation of the trunk, by clamping the common carotid arteries and the internal and external jugular veins simultaneously low in the neck, having first ligated the vertebral arteries at the point where they enter their canals in the transverse processes of the cervical vertebrae.

strictor influence an insufficient volume of blood reaches the heart. By means of transfusion, however, it was possible to raise the blood pressure and maintain the circulation at a point permitting manometric record of further change in the pressure, and under such circumstances it is found that the injection of horse serum into a sensitized dog causes a definite fall in pressure, demonstrating conclusively that the essential phenomenon of anaphylaxis may be brought about independently of the cerebro-medullary and the spinal centers.

3. While the experiments on decapitated animals and on those with spinal cord destroyed and with vagi and cervical sympathetic nerves severed have shown that the usual effect on the blood pressure may be produced independently of the central action, such experiments have not of course shown that the central vasomotor mechanism does not perhaps play some part in the symptom complex presented by intact animals.

With the object of ascertaining the possibility of a central action in anaphylaxis, without the complication of the known peripheral effects, we were obliged to devise a means by which the horse serum might be introduced into the cerebral circulation, but not reach the circulation of the body. For this purpose a special operation was perfected, the details of which are described elsewhere,[1] whereby all vascular communication between the trunk and head was obliterated and the circulation in the head and neck maintained independently by transfusion from the carotid artery of a normal animal, the outflow from the recipient being through a free opening in the external jugular vein. Following a short period of irregularity due to the temporary changes in the cerebral circulation incidental to the technic of isolation, the general blood pressure of the recipient is maintained at a normal level, and horse serum may be introduced into the cerebral or into the peripheral circulations exclusively without any of the injected material reaching the circulation of the other portion of the animal.

When, under such circumstances, horse serum is introduced through the anastomosing carotid artery into the cerebral circulation of a sensitized animal, a slight transient lowering of the blood pressure is produced, but it is not the typical depression of anaphy-

[1] A. B. Eisenbrey, *Proc. Soc. Exper. Biol.*, 1910, 7 (in press).

lactic shock in intact animals. The action is prompt, but the depression, to give the figures in one experiment, is only 16 mm. Hg with return to normal level in 23 seconds. If, some minutes after recovery from this effect, the serum is injected into the circulation of the trunk, it produces a lowering of the blood pressure to a level which corresponds to that in the typical condition of anaphylactic shock in the intact animal, for example, in the experiment quoted a fall of 74 mm. Hg with the low level persisting.

In view of the well-known fact that when anaphylactic shock is once produced further injections of the serum have no effect, the results in the above experiments show not only the completeness of the isolation of the cerebral circulation from that of the trunk but also that the peripheral influence is the essential factor in the production of the change in blood pressure so characteristic of anaphylaxis.

It was apparent, therefore, that the action was in the largest degree peripheral, either on the nerve endings or on the muscle of the vessels themselves, which is in accord with the observations of Biedl and Kraus concerning the influence of barium chloride in modifying the blood pressure phenomena of anaphylactic shock.

A number of pharmacological experiments were then made to still further localize the action. These are based on the experiments of Dixon[1] and Brodie and Dixon[2] with apocodeine. This substance in very large doses paralyzes vasomotor nerve endings; and "when its action is complete pilocarpine, physostigmine, and adrenalin have no effect on the blood vessels or blood pressure, whilst barium and digitalis will still constrict the blood vessels and raise blood pressure. The first three drugs, it is therefore assumed, act on nerve endings and the latter two directly on muscle."

As the low pressure of anaphylaxis both in its level and in permanence resembles closely that due to apocodeine we have tested the effect of adrenalin and barium in the hope of finding analogous mechanisms. The results are not conclusive. While barium causes an increase in pressure,[1] adrenalin does likewise; the latter, however, is but slight and never equal, even if percentile values are considered,

[1] *Jour. of Physiol.*, 1903, 30, p. 97. [2] *Ibid.*, p. 476.

[1] We have also confirmed the observation of Biedl and Kraus that the administration of barium chloride to a sensitized animal before giving the toxic dose modifies or prevents the characteristic fall in pressure.

to the normal adrenalin reaction. Assuming therefore that adrenalin acts through the nerve endings, it is evident that in anaphylactic shock altho these structures are not completely paralyzed, their activity is greatly diminished not only as shown by the slight adrenalin action but also by the fact that splanchnic stimulation causes a smaller rise in pressure than in the normal animal. Nitroglycerin, which according to Dixon and Brodie acts only on the vessel muscle, also causes a further fall in pressure, as does also to a slight extent dog's urine, which as we have shown elsewhere,[2] apparently lowers blood pressure by an action on the nerve endings. It is therefore evident that altho this condition of low pressure is due in greatest part, at least, to a disturbance of the peripheral vasomotor mechanism, it is not possible to say that the function of either the nerve endings or the muscle is completely in abeyance. The pharmacological experiments indicate, however, that the nerve endings bear the brunt of the injury. This view is supported by experiments with apocodeine. If a sensitized dog under the influence of apocodeine received an injection of serum, a further depression of the circulation was not obtained. In fact, the blood pressure was raised a few millimeters by the mechanical effect of the injection and remained so for a period of about one minute, gradually returning to a point slightly below the original level after two minutes.

SUMMARY

1. Anaphylaxis in the dog is characterized subjectively by a sudden and persistent fall in blood pressure followed by objective symptoms referable to cerebral anemia.

2. Exsanguination and transfusion experiments demonstrate that the disturbance is due to a reaction in the fixt cells and not to changes occurring primarily or secondarily in the circulating blood.

3. Experiments in which the peripheral and central vasomotor mechanisms have been separated by various methods (section, decapitation, destruction of the cord, and isolated circulation) show that the chief influence is exerted on the peripheral vaso-motor system.

4. Pharmacological experiments point to an influence on the nerve endings rather than to one on the muscle of the vessels.

[2] *Amer. Jour. of Physiol.*, 1910, 26, p. 26.

THE INFLUENCE OF CHLORAL HYDRATE ON SERUM ANAPHYLAXIS.*†

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In a preliminary communication read before the Society for Experimental Biology and Medicine in February 1908, we reported the following:

We found that by injecting a solution of chloral hydrate which was just sufficient in strength to produce hypnosis, fully 75 per cent of all serum-sensitized guinea-pigs were completely protected from a second injection of serum into the peritoneal cavity, whereas 90 per cent of controls died. We believed that with improved technic in the dosage of chloral hydrate it would be possible to protect 90 per cent of all fully sensitized guinea-pigs. By "fully sensitized," we meant that at least three weeks' or a month's time should have elapsed before the second injection of serum into guinea-pigs which had received horse serum alone (1/100 to 1/500 c.c.); at least seven or eight weeks should have elapsed before the second injection. By allowing the above interval of time to elapse, over 90 per cent of our controls died within an hour, most of them within 20 minutes.

We found that the dose of the chloral hydrate per gram weight of the animal was not a simple ratio, no fixt amount could be stated, much depending upon the individual idiosyncrasy of the animal. Approximately 75 milligrams of the drug to a 250 gram guinea-pig, and 100 milligrams to a 300 gram guinea-pig produce the degree of hypnosis desired.

We used a fresh 10 per cent solution of chloral hydrate, carefully measured out the required amount into a small sterile beaker, and added an equal amount of sterile water. This diluted solution was injected into the muscles of the thigh of the animal, half into one leg and half into the other. After 20 to 30 minutes, the needle was inserted into the peritoneal cavity. This caused muscular twitching and slight movement of the head. This indicated the proper degree of hypnosis. The injection of 5 c.c. serum was then given and the animal kept in a warm room. No symptoms appeared and the sleep was undisturbed. After 2½ to 3½ hours have elapsed, the animal slowly recovers from the effect of the drug. No symptoms or ill effects have been observed in any of the animals. Observations have been followed for over two weeks after treatment. The animal, after the effects of the drug have disappeared, will react with characteristic symptoms of anaphylaxis if given a third injection of the serum. We injected only 24, 48, and 72 hours after recovery from the effects of the drug.

If the dose of chloral hydrate has not been sufficient, the insertion of the needle into the peritoneal cavity will cause marked muscular movements, raising of the head, and an attempt to regain its feet. Under these conditions if the serum is injected the animal will die of anaphylaxis.

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† Read before the American Society of Biological Chemists, Baltimore, December, 1908.

On the other hand, if the animal shows no muscular twitchings whatever, the dose of chloral has probably been too large. We wish to emphasize the fact that great care must be used not to overdose the sensitized guinea-pig with chloral hydrate, altho a sensitized as well as a normal guinea-pig will recover from a large dose, considerably more than the amount mentioned above. Apparently the combined effects of the drug and the serum in a sensitized animal produce a deeper hypnosis than the drug when given alone.

Further work, following along the lines of the above preliminary report, has fully confirmed our earlier observations.

We realized that for our work it was essential to determine the best method for sensitizing the guinea-pigs so that on receiving the second injection of horse serum intraperitoneally the animals would surely die from anaphylaxis. We therefore injected several series of guinea-pigs subcutaneously with from 0.01 c.c. to 0.001 c.c. of horse serum. After 3 to 6 weeks, we found that in none of the series did 100 per cent of deaths occur when the animals were injected with 5 c.c. horse serum intraperitoneally. In our later work, we have used only those guinea-pigs which had survived the routine testing of antitoxin. After a lapse of three weeks or longer, these animals, in our experience, succumb almost without exception to the second injection of 5 c.c. horse serum intraperitoneally.

In this later work, we have given the second injection of serum only to those sensitized guinea-pigs which showed favorable hypnosis, indicated by only slight muscular twitchings when the needle is inserted into the peritoneal cavity. If muscular movements are marked we do not inject. In this case, we allow the animal to recover from the effects of the drug, and next day increase the dose about one-fifth. This usually brings about the optimum hypnosis in about 25 to 30 minutes. In following this method we have frequently protected 100 per cent, while 100 per cent of the controls died.

In our work thus far it appears as tho the protection chloral hydrate affords a sensitized guinea-pig depends on the degree of sensitization. If we accept the hypothesis of Vaughan, this may be expressed as the amount of ferment or zymogen present in the sensitized guinea-pig, its supposed degree of activity or power in freeing or splitting off definite quantities of a poisonous substance from the serum given in the second injection accounting for the symptoms of anaphylaxis.

This appears to be the case in the following table. The eight

guinea-pigs were well under the influence of the drug when the second injection was given. Guinea-pigs 393, 702, and 146 died in 15, 30, and 20 minutes respectively with typical symptoms of anaphylaxis, the heart continuing to beat after respiration ceased.

TABLE 1.

Intramuscular Injection of Chloral Hydrate Followed by 5 c.c. Normal Horse Serum.

G.-P. No.	Weight in Grams	Previous Treatment	Interval in Days	Chloral Hydrate in Mg.	Second Injection Intraperitoneally	Symptoms	Results
330....	310	0.56 c.c. toxin + 1/500 c.c. antitoxic horse serum	42	125	5 c.c. normal horse serum	None	Recovered
393....	265	" "	42	90	"	Typical	Death within 15 min.
355....	260	0.56 c.c. toxin + 1/300 c.c. antitoxic horse serum	28	90	"	None	Recovered
376....	260	" "	28	85	"	"	"
358....	260	0.56 c.c. toxin + 1/350 c.c. antitoxic horse serum	32	85	"	"	"
363....	280	" "	32	100	"	"	"
702....	395	0.55 c.c. toxin + 1/300 c.c. antitoxic horse serum	62	180	"	Typical	Death within 30 min.
146....	335	" "	62	150	"	"	Death within 20 min.
313....	295	0.56 c.c. toxin + 1/500 c.c. antitoxin horse serum	42		"	"	Death within 18 min.
372....	260	" "	42		"	"	Death within 25 min.
595....	240	0.56 c.c. toxin + 1/200 c.c. antitoxic horse serum	28		"	"	Death within 12 min.
696....	240	" "	28		"	"	Death within 17 min.
513....	230	0.56 c.c. toxin + 1/300 c.c. antitoxic horse serum	33		"	"	Death within 12 min.
347....	260	" "	33			"	Death within 33 min.

Many series of sensitized guinea-pigs were given chloral hydrate intramuscularly followed by the second injection of horse serum intraperitoneally, and in almost all cases one or two guinea-pigs of each series would die with typical symptoms of anaphylaxis, even tho the degree of hypnosis seemed sufficient. This leads us to believe that in such guinea-pigs more poison was elaborated or split off from the serum in a given time than in those that were protected; for example say, if just a single fatal dose of poison is split off, the chloral will protect, but if two or more fatal doses are split off, the chloral cannot protect the animal. A quantitative relationship may exist.

In Table 2 the controls died promptly, while those which received the chloral were protected.

Guinea-pigs 60 and 202 had received 180 and 160 milligrams chloral, respectively, the day before. They then weighed 410 and 370 grams. This amount, however, proved insufficient to produce the desired hypnosis. They were allowed to recover, and the following day weighed 370 and 335 grams respectively. They were again given the same amount of the drug, 180 and 150 milligrams. In 25 minutes favorable hypnosis was produced. The 5 c.c. of horse serum were then injected. The following day these two guinea-pigs were chloroformed and autopsied. No definite lesions were found.

TABLE 2.

INTRAMUSCULAR INJECTION OF CHLORAL HYDRATE FOLLOWED BY 5 C.C. NORMAL HORSE SERUM.

G.-P. No.	Weight in Grams	Previous Treatment	Interval in Days	Chloral Hydrate in Mg.	Second Injection Intraperitoneally	Symptoms	Results
60....	370	0.56 c.c. toxin + 1/350 c.c. antitoxic horse serum	85	180	5 c.c. normal horse serum	Slight twitching of ears	Recovered
202....	335	0.56 c.c. toxin + 1/750 c.c. antitoxic horse serum	82	160	"	None	"
240....	370	0.56 c.c. toxin + 1/400 c.c. antitoxic horse serum	74	170	"	"	"
777....	325	0.56 c.c. toxin + 1/800 c.c. antitoxic horse serum	68	130	"	Slight twitching of ears	"
748....	345	0.56 c.c. toxin + 1/250 c.c. antitoxic horse serum	68	155	"	"	"
438....	375	0.56 c.c. toxin + 1/200 c.c. antitoxic horse serum	85		"	Typical	Death within 20 min.
143....	360	0.56 c.c. toxin + 1/300 c.c. antitoxic horse serum	82		"	"	Death within 13 min.
251....	395	0.56 c.c. toxin + 1/250 c.c. antitoxic horse serum	81		"	"	Death within 20 min.
256....	360	0.56 c.c. toxin + 1/750 c.c. antitoxic horse serum	78		"	"	Death within 32 min.
25....	290	0.56 c.c. toxin + 1/900 c.c. antitoxic horse serum	50		"	"	Death within 25 min.

Guinea-pigs 240, 777, and 748, three days later, again received 5 c.c. horse serum intraperitoneally; slight but definite symptoms appeared in the first two animals after about 15 minutes. These symptoms subsided in about 30 minutes. In the third animal questionable symptoms were noted. Other sensitized guinea-pigs that had been protected from the second injection of serum with chloral were also reinjected; about 75 per cent showed slight but definite symptoms.

CHLORAL HYDRATE INJECTED INTRAMUSCULARLY, FOLLOWED BY NORMAL HORSE SERUM INTRACARDIACALLY.

Sensitized guinea-pigs were given chloral hydrate in the same manner as mentioned above. Those animals which showed favorable hypnosis were injected directly into the heart with 0.25 c.c. of normal horse serum. After about one-half minute all the animals showed typical symptoms of anaphylaxis, dying within three to four minutes. All the controls also died within three to four minutes. This experiment was repeated several times with the same results; none of the guinea-pigs were protected.

All these animals were autopsied. We found no deaths due to direct hemorrhage. Gross lesions were found in other organs.

TABLE 3.

INTRAMUSCULAR INJECTIONS OF CHLORAL HYDRATE FOLLOWED BY NORMAL HORSE SERUM INTRACARDIACALLY.

G. P. No.	Weight in Grams	Previous Treatment	Interval in Days	Chloral Hydrate in Mg. Intramuscularly	Second Injection Horse Serum Intracardiacally	Symptoms	Result	Autopsy
353	290	0.56 c.c. toxin + 1/300 c.c. antitoxic horse serum	21	125	0.25 c.c.	Typical	Death within 3 min.	No hemorrhage in pericardium
535	330	0.56 c.c. toxin + 1/400 c.c. antitoxic horse serum	51	160	"	"	Death within 4 min.	" "
107	300	"	20	140	"	"	"	"
237	280	"	20	120	"	"	"	"
218	520	0.56 c.c. toxin + 1/100 c.c. antitoxic horse serum	29		"	"	Death within 3 min.	"
272	300	0.56 c.c. toxin + 1/400 c.c. antitoxic horse serum	20		"	"	"	"
100	275	"	20		"	"	Death within 4 min.	"

In seeking an explanation for these results, we came to the conclusion that a certain optimum content of the drug in the circulation was essential to protect the animal; that possibly the lipoids played an important rôle in anaphylaxis; that if we injected a sufficient amount of chloral hydrate directly into the circulation, we would obtain a loose physico-chemical combination with the vitally important lipoids of the cells and in so doing change their normal relationship to the other cell constituents, through which an inhibition of the entire cell chemism would result. With this in mind, we injected sensitized guinea-pigs weighing between 275 and 300 grams with 30

milligrams of the drug directly into the heart. Even before completion of the injection, the animals were under the influence of the drug. After two to four minutes the dose was repeated again into the heart. After again allowing two to four minutes to elapse 0.25 c.c. of horse serum was injected into the heart.

Chloral hydrate given in this manner protects about 75 per cent of the sensitized guinea-pigs from the second injection when made directly into the heart.

CHLORAL HYDRATE INJECTED INTRACARDIACALLY FOLLOWED BY NORMAL HORSE SERUM.

Solutions of chloral hydrate were given intracardiacally in divided doses to sensitized guinea-pigs. When complete hypnosis was produced 0.25 c.c. of normal horse serum was given in the same manner.

The dilutions for injections were such that not more than 1.5 c.c. were given at one time. These injections were given very slowly so that the danger from possible dilation of the heart could be largely ruled out. Also, since intracardiac injections very frequently result in injury to the heart muscle, causing hemorrhages, and since the whole dose does not always enter the heart, experiments were repeated many times. Autopsies were performed in all cases in which death occurred in order to exclude mechanical injury with resulting hemorrhage as the cause of death.

In our preliminary work the selected dose of the drug, which was about one-half of the amount for an intramuscular dose, was given intracardiacally at one injection. This, however, frequently caused the death of the animal. We, therefore, resorted to divided doses at short intervals. With this method few deaths resulted from chloral injections.

In Table 4, guinea-pig 139 received 45 milligrams chloral hydrate intracardiacally; even before the injection was completed the animal was under the influence of the drug. The respiration was deep and irregular for about 10 seconds, then short and rapid for about one minute. Three minutes after the first injection, 40 milligrams more of the drug were given; the same irregular respirations were observed. Two minutes after the second injection of the drug, 0.25 c.c. normal horse serum was given. A few twitchings at the nose were noticed. Thirty minutes later the animal showed signs of recovery from the effects of the drug and 2½ hours later complete recovery was effected. Guinea-pig 995 received 40 milligrams chloral hydrate. The animal was under the influence of the drug at the completion of the injection. The respiration was deep and

irregular for about 10 seconds, then short and rapid. Two minutes after the first injection 40 milligrams more of the drug were given. Two minutes later 0.25 c.c. normal horse serum was given. No symptoms were noticeable. About 2½ hours later the animal had recovered from the effects of the drug.

Guinea-pig 16 received 35 milligrams chloral hydrate. The animal was under the influence of the drug at the completion of the injection. Four minutes later 30 milligrams more of the drug were given. Three minutes later 0.25 c.c. of normal horse serum was given. No symptoms were noticeable. About two hours later the animal had recovered from the effects of the drug.

Guinea-pig 8 struggled considerably while receiving the 30 milligrams of the chloral hydrate, so that most of the drug did not enter the heart. The animal could run around immediately after the injection. Four minutes later the animal was still walking around. We then gave it 35 milligrams more of the drug in the heart this time. The animal was then under the influence of the drug. Four minutes later 0.25 c.c. normal horse serum was injected. About one-half minute later typical symptoms developed, the animal dying within four minutes. Autopsy showed no direct hemorrhage. It appears in this case as tho an insufficient amount of chloral had entered the circulation.

Guinea-pig 24 struggled considerably while receiving the 30 milligrams of chloral. Most of the drug did not enter the heart. The animal could run around. Having in mind the result of the inoculations of guinea-pig 8, we decided to wait 20 minutes before injecting again. At the end of 20 minutes the animal was in a partial stupor. Thirty milligrams of chloral were given, the animal being under the influence of the drug at the completion of the injection. Four minutes later an additional 30 milligrams of the drug were given. This was followed three minutes later with 0.25 c.c. normal horse serum. No symptoms were noticeable. About three hours later the animal recovered from the effects of the drug.

Twenty-two hours later, guinea-pigs 139, 995, 16, and 24 received an additional 0.5 c.c. normal horse serum intracardiacally. All showed typical symptoms after about one-half minute. Respiration ceased within three minutes. The heart continued to beat after respiration had ceased. Autopsies showed none of the deaths due to direct hemorrhage. These experiments were repeated many times with similar results.

The fact that those sensitized guinea-pigs, which, while under the influence of the drug, were protected from the second injection of serum, are still sensitized, leads us to the following conclusions. Certain parts of the serum necessary to cause anaphylaxis, when injected into the circulation of a sensitized pig, must be rapidly destroyed or eliminated from circulation. Or these parts may combine with cells not vitally important to the immediate life of the animal. If this be the case, the ferment or zymogen, in those protected, sensitized guinea-pigs, has not all been utilized in destroying or splitting the serum.

In other words, the animal is not in most cases vaccinated, but

TABLE 4.

INTRACARDIAC INJECTIONS OF CHLORAL HYDRATE FOLLOWED BY 0.25 C.C. NORMAL HORSE SERUM. TIME INTERVAL BETWEEN INJECTIONS 2 TO 4 MINUTES.

Guinea-Pig No.	Weight in Grams	Previous Treatment	Interval in Days	Chloral Hydrate in Mg. Intra-cardiacally	Second Injection Horse Serum Intracardiacally	Symptoms	Result	Autopsy
139	380	0.73 c.c. toxin + 1/500 c.c. antitoxic horse serum	50	1st inj. 45 mg. 2d " 40 "	0.25 c.c.	After 10 min. slight scratches at nose	Recovered	
195	340	0.73 c.c. toxin + 1/1200 c.c. antitoxic horse serum	30	1st " 40 " 2d " 40 "	"	None	"	
16	310	0.73 c.c. toxin + 1/100 c.c. antitoxic horse serum	50	1st " 35 " 2d " 30 "	"	"	"	
8	335	0.73 c.c. toxin + 1/600 c.c. antitoxic horse serum	50	1st " 30 " missed heart 2d inj. 35 mg.	"	Typical	Death within 4 min.	No hemorrhage in pericardium
24	305	0.73 c.c. toxin + 1/450 c.c. antitoxic horse serum	30	1st " 30 " missed heart 2d inj. 30 mg. 3d " 30 "	"	None	Recovered	
13	300	0.73 c.c. toxin + 1/500 c.c. antitoxic horse serum	50		"	Typical	Death within in 3 min.	No hemorrhage in pericardium
42	320	0.73 c.c. toxin + 1/1250 c.c. antitoxic horse serum	30		"	"	"	"
33	360	0.73 c.c. toxin + 1/400 c.c. antitoxic horse serum	30		"	"	"	"

TABLE 5.

INTRACARDIAC INJECTIONS OF CHLORAL HYDRATE FOLLOWED BY 0.25 C.C. NORMAL HORSE SERUM. TIME INTERVAL BETWEEN INJECTIONS 12 TO 15 MINUTES.

Guinea-Pig No.	Weight in Grams	Previous Treatment	Interval in Days	Chloral Hydrate Intracardiacally	Second Injection Horse Serum Intracardiacally	Symptoms	Result	Autopsy
168	300	0.73 c.c. toxin + 1/1400 c.c. antitoxic horse serum	18	1st inj. 30 mg. 2d " 30 " 3d " 30 "	0.25 c.c.	Typical	Death within 5 min.	No hemorrhage in pericardium
11	290	0.73 c.c. toxin + 1/1700 c.c. antitoxic horse serum	18	1st inj. 30 mg. 2d " 30 " 3d " 20 "	"	"	"	"

will react with typical symptoms, followed by death, if, after recovery from the effects of the drug, an additional 1.5 c.c. of horse serum is given intracardiacally.

We wish to emphasize that from our experiments a certain optimum content of the drug in the circulation is essential to protect the animal. The drug is rapidly destroyed or eliminated from the circulation. This we believe can be shown by allowing 12 to 15 minutes to elapse between the intracardiac injections of the drug and 12 to 15 minutes to elapse before injecting the serum. Chloral hydrate given in this manner, with this time allowance, will not protect sensitized guinea-pigs from the second injection intracardiacally.

CHLORAL HYDRATE INJECTED INTRACARDIACALLY FOLLOWED BY 0.25 C.C. NORMAL HORSE SERUM INTRACEREBRALLY.

Sensitized guinea-pigs were trephined and then given divided doses of chloral hydrate in the manner mentioned above. The time allowance between injections of the drug was from two to four minutes. One quarter c.c. of normal horse serum was then given intracerebrally after the method of Besredka. About 75 per cent of the sensitized guinea-pigs tested in this manner were protected from the second injection intracerebrally.

TABLE 6.

INTRACARDIAC INJECTIONS OF CHLORAL HYDRATE FOLLOWED BY 0.25 C.C. NORMAL HORSE SERUM INTRACEREBRALLY. TIME INTERVAL BETWEEN INJECTIONS 2 TO 4 MINUTES.

G.-P. No.	Weight in Grams	Previous Treatment	Interval in Days	Chloral Hydrate Intracardiacally	Second Injection Horse Serum Intracerebrally	Symptoms	Results
43....	400	0.73 c.c. toxin+ 1/750 c.c. antitoxic horse serum	22	1st inj. 45 mg. 2d " 45	0.25 c.c.	None	Recovered
2....	395	0.73 c.c. toxin+ 1/500 c.c. antitoxic horse serum	32	1st " 40 " 2d " 40 "	"	"	"
26....	295	0.73 c.c. toxin+ 1/400 c.c. antitoxic horse serum	37	1st " 35 " 2d " 35 "	"	"	"
7....	415	0.73 c.c. toxin+ 1/500 c.c. antitoxic horse serum	32	1st " 45 " 2d " 45 "	"	"	"
29....	275	0.73 c.c. toxin+ 1/300 c.c. antitoxic horse serum	40		"	Typical	Death within 5 min.
333....	340	"	32		"	"	"
934....	380	"	38		"	"	"

With the different methods of administering the second injection of horse serum into sensitized guinea-pigs, which were favorably

under the influence of chloral hydrate, we were able to protect about 75 per cent. In our preliminary communication we stated that we believed that with improved technic in the dosage of chloral hydrate it would be possible to protect 90 per cent of all fully sensitized guinea-pigs.

Now, however, we have come to the conclusion that, if the smallest dose of serum which will just kill any animal of a given series of sensitized guinea-pigs regularly, be injected into the remainder of the animals of the same series of sensitized guinea-pigs, when properly under the influence of chloral hydrate, protection will be afforded in practically all cases.

STUDIES ON INHIBITION, ATTENUATION, AND REJUVENATION OF BACILLUS COLI.*

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THERE has been much discussion and uncertainty as to the delicacy and relative value of dextrose broth[1] and lactose bile[2,3,4] in testing water of fairly good quality.[5,6,7] The present investigation was undertaken with a view to throwing light upon this subject.

Comparison has been made between plain broth, dextrose broth, lactose broth, lactose bile, and other liquid media under greatly varying conditions, artificial and natural. In the case of plain broth, 1 c.c. was transplanted at the end of 24 hours into lactose bile and in some series into dextrose broth. In other series dextrose broth was transplanted into lactose bile after 24 or 48 hours. In a few experiments bile was transplanted into bile.

EXPERIMENTS UNDER ARTIFICIAL CONDITIONS.

Two sets of experiments were devised and carried out in different years with practically the same results. The object was to ascertain whether dextrose broth would show gas formation in higher dilution than would lactose bile. The first series was carried out with well water, the second with surface water. In each case, the water was not sterilized but was tested with both dextrose broth and lactose bile to prove the absence of gas-forming bacteria. The surface water was in addition incubated at 37° C. for two days before testing for gas formers. A pure culture of B. coli was then introduced into the bottles of water and the samples kept at three temperatures, 37° C., 20° C., and 8° C. Daily tests were made upon each sample with dextrose broth and with lactose bile in quantities of 10 c.c., 1 c.c., 0.1 c.c., 0.01 c.c., etc., using a sufficient number of dilutions by tenths always to obtain the zero point, until gas formation was no longer obtained in 10 c.c. of the water. The accompanying charts, A and B, show the results of these experiments.

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As no gas formers were originally present, the formation of any amount of gas, no matter how small, in the dextrose broth indicated B. coli. Unsterilized water was used in order to approach natural conditions and allow of interference with the tests for B. coli by the water bacteria present. The tests were planned to show also any

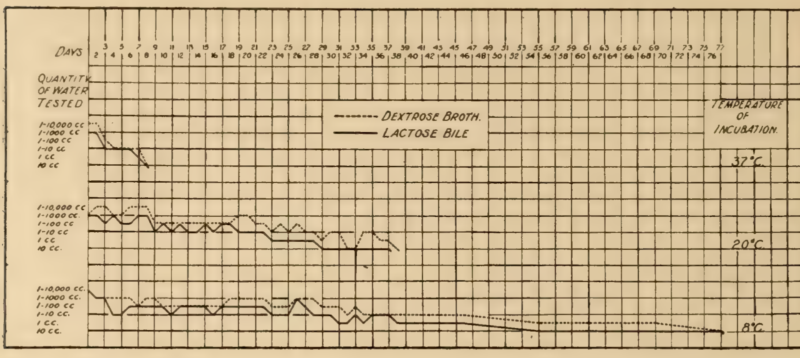

CHART 1.—Comparison, made with dextrose broth and lactose bile, of tests for B. coli inoculated into unsterilized well water.

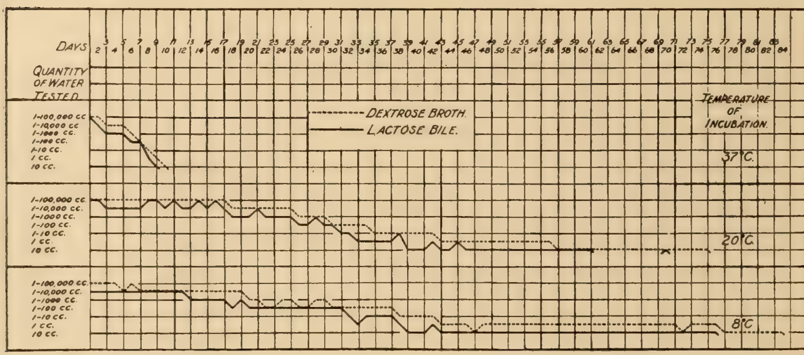

CHART 2.—Comparison, made with dextrose broth and lactose bile, of tests for B. coli inoculated into unsterilized surface water.

difference that might exist between dextrose broth and lactose bile in indicating B. coli when vigorous and when attenuated. The experiments likewise serve to demonstrate the viability of B. coli at the three temperatures and under the existent conditions.

Viability.—It will be seen from the charts that the B. coli quickly died out in the samples kept at 37° C., lasting only 8 to 10 days.

At 20° C. and 8° C., B. coli lived much longer, 38 to 75 days at 20° C. and 77 to 84 days at 8° C. Even then its presence was detected by a more delicate medium, liver broth,[8] recently devised in this laboratory.

It is important to know in regard to samples of water which have necessarily been some time in transit whether the test for B. coli is trustworthy as evidence of the presence and amount of pollution. The charts seem to indicate that samples shipped without ice, unless they should become considerably warmed in transit, may yield good evidence even if a week old.[9]

Bacteria present.—In the first set of experiments (Chart 1), the samples were plated daily in litmus-lactose-agar and in gelatin. The well water originally showed 12 bacteria per c.c. on gelatin. After inoculation with B. coli, litmus-lactose-agar gave counts per c.c. for the first few days, before the bacteria had become attenuated, as follows:

Sample incubated at 37° C.—14,000, 2,000, 200; at 20° C. 6,000, 8,000, 5,900, 3,200; at 8° C.—80,000, 4,000, 3,700, 900, 1,200.

Comparing these counts with the dilutions showing gas formation in dextrose broth and lactose bile it will be seen that the number of bacteria giving a test are as follows:

Dextrose broth, series at 37° C.—14, 1, 2; at 20° C.—6, 1, 1, 3; at 8° C.—8, 4, 4, 1, 1; average 4.

Lactose bile, series at 37° C.—140, 2, 20; at 20° C.—6, 8, 59, 3; at 8° C.—8, 4, 4, 90, 120; average 39.

While the colon bacilli were gradually dying out, the water bacteria were rapidly increasing, and also new species were introduced from time to time because of the daily slight exposure of the bottles to the air in testing. In the series at 37° C. the agar plates showed 30,000 on the fourth day, due to water forms which grow at 37° C. In a few days all bacteria had disappeared from this sample with the exception of a few bacteria growing on gelatin at 20° C. In the series at 20° C. the water bacteria as shown by gelatin increased to a maximum of 242,000 per c.c. on the fifth day, dropped to 700 on the ninth day, rose to 66,000 on the tenth day and then fluctuated between 1,500 and 13,000 for the remainder of the time. At the end they were slowly increasing and had reached 4,000. In the

series at 8° C. the water bacteria reached 10,000 on the seventh day, dropped to 900 on the ninth day, reached a maximum of 20,000 on the tenth day, increased from 6,000 on the eleventh to 13,000 on the sixteenth and from 3,500 on the seventeenth to 17,000 on the twenty-fourth day. B. liquifaciens fluorescens made its first appearance on the nineteenth day and continued thereafter. From the twenty-fifth to the twenty-eighth day there were about 12,000 bacteria present, from the thirty-first to the thirty-sixth 5,000, thirty-seventh to fortieth 15,000, decreasing to 3,000 on the forty-fourth day. There was no very apparent relationship between the fluctuations of water bacteria and the tests for B. coli. Occasionally a sudden increase in the water bacteria was accompanied by a corresponding decrease in the test for B. coli.

Dextrose broth.—As a presumptive test for B. coli, dextrose broth has been limited to certain empirical rules, namely, gas production 25 per cent to 70 per cent and carbon dioxide 25 per cent to 40 per cent. Extended experience has shown this test to be very unsatisfactory for several reasons:[7, 10, 11, 12, 13] (1) several other species will give the formula; (2) many other species produce gas in dextrose broth;[10] (3) other bacteria interfere with the test, causing numerous anomalies.[12]

A most striking feature of the above series of experiments was the complete failure of the formula[7, 13] in the presence of no other gas former than B. coli. This was probably due to interference of the water bacteria. Out of 818 tests which showed gas formation, only 474, or 58 per cent, met the formula. Out of 259 sets of dilutions only 62, or 24 per cent, failed to show one or more anomalies.

Lactose bile.—In contrast to dextrose broth, the bile gave 599 positive tests for B. coli, i.e., over 25 per cent total gas within three days. Only three tests gave gas which did not reach 25 per cent. There was not a single anomaly, i.e., each set of dilutions showed a consecutive series of positive tests.

The rapidity with which the gas appeared in the bile also gave some idea of the degree of attenuation of the B. coli. A study of the details of the series of experiments represented in Chart 1 brought out the following features:

For six days every test in the bile was positive in 24 hours. After

the sixth day only two tests became positive in 24 hours, one on the eighth day and one on the twenty-fifth day. On the ninth day occurred the first test that required three days to develop and that was the highest dilution of a set. On the twelfth day three-day tests were frequent. On the sixteenth day for the first time every test required three days, although two-day tests were frequent to the end. On the thirty-first and thirty-eighth days occurred the three tests which failed to show 25 per cent gas in three days.

Comparative delicacy.—The series of experiments represented in Chart 1 were carried out with the lactose bile as first described. In the series of Chart 2 lactose bile with 1 per cent peptone[14] added was used, since it had been learned in the meantime that the addition of peptone hastened the production of gas and increased its quantity, rendering the test considerably more delicate. It may be stated in passing that the addition of Nährstoff Heyden[15] and of fresh meat infusion to lactose bile were tried, but failed to improve its delicacy in any respect. Peptone alone improved it. In the present experiments the results did not show the usual improvement of lactose bile with peptone over lactose bile except in a very slight degree.

A comparison between dextrose broth, considering any gas formation as a positive test, since B. coli only was present, and the test as given by lactose bile and by lactose bile with peptone is given as follows:

Dextrose broth gave positive tests more delicately than

	Lactose Bile		Lactose Bile with Peptone	
Two dilutions* greater..........	14 times	—11%	10 times	— 6%
One " "	81 "	66 "	124 "	73 "
No " "	28 "	23 "	35 "	21 "
	123		169	

* All dilutions are in tenths.

Allowing double weight for two dilutions the dextrose broth was one dilution ahead of lactose bile 109 times out of 123, i.e., 89 per cent, and similarly ahead of lactose bile with peptone 144 times out of 169, i.e., 85 per cent. Considered from another standpoint it required about 10 bacilli to produce a test in lactose bile for every 1 bacillus necessary to produce gas in dextrose broth under the conditions of these experiments. This was due to the inhibitive action

of the bile salts, since lactose broth showed gas in as high dilution as did the dextrose broth when comparisons were made, i.e., the sugar, lactose, was not the inhibiting agent.

Rejuvenation tests.—Toward the end of these experiments, while B. coli was attenuated, additional comparisons were made by planting dilutions into plain broth and transplanting into lactose bile at the end of twenty-four hours. Again comparisons were made using dextrose broth made with fresh beef infusion, instead of Liebig's beef extract as used in the regular dextrose broth. The results of these comparisons are seen as follows:

Rejuvenation in plain broth gave a test for B. coli:

One dilution greater than dextrose broth (Liebig's extract)	8 times
No " " " " " " "	9 "
One " less " " " " "	3 "
	20
One dilution greater than dextrose broth (beef infusion)	1 time
No " " " " " " "	16 "
One " less " " " " "	3 "
	20
Three dilutions greater than lactose bile	2 times
Two " " " " "	4 "
One " " " " "	11 "
No " " " " "	3 "
	20

The results of these tests were averaged by multiplying a three-dilution advantage by three, a two-dilution advantage by two, adding the single dilution advantage and subtracting the dilution disadvantage. The results thus compared showed that rejuvenation in plain broth in these twenty experiments gave a test on an average $1\frac{1}{4}$-dilution higher than lactose bile, $\frac{1}{4}$-dilution higher than dextrose broth (Liebig's extract), and $\frac{1}{10}$ dilution less than dextrose broth (fresh beef infusion). It is apparent that the difference between plain broth and dextrose broth was due to the presence and absence of fresh meat infusion and not to the presence of sugar. Hence by planting dilutions of water into dextrose broth made with fresh meat infusion and, as soon as gas formation appears, transplanting into lactose bile, a larger number of tests for B. coli may be obtained than by using lactose bile alone. This excess B. coli is usually,

however, attenuated and of less importance than vigorous B. coli which indicates fresh contamination.

EXPERIMENTS WITH ROUTINE SAMPLES OF WATER UNDER NATURAL CONDITIONS.

The previous experiments were conceived for the purpose of keeping strict control over all conditions with an exact knowledge of the gas former present. Altho the samples were unsterilized, thus allowing for the presence of large numbers of water bacteria, yet the experiments could not fail to be artificial in character.

In order to supplement and test under a wider range of natural conditions the information gained, daily comparisons were made upon Manhattan and Brooklyn waters between dextrose broth and lactose bile in one year and between lactose bile, lactose bile with peptone, and dextrose broth transplanted to lactose bile with peptone, in another year, during the seasons when B. coli is prevalent. Some 60 comparisons were also made with plain broth transplanted to bile.

The results of comparisons upon the waters of the two boroughs have been kept separate, and contrary to expectation the bile appeared to the best advantage in testing the water supplied to Manhattan. This was true in every comparison of both years. The Manhattan supply has long storage and B. coli should be more attenuated than in the Brooklyn water. The reason for these results probably rests with a difference in the water bacteria present which interfere with the gas production or growth of B. coli.

The following table gives the results of comparison for the first year between dextrose broth and lactose bile. With the dextrose

TABLE I.

		Positive Presumptive Tests for B. Coli		Total Gas Formers
		Lactose Bile	Dextrose Broth (formula)	Dextrose Broth 5% Gas and Over
Manhattan supply (399 samples)	0.1 c.c.	16— 4%	20— 5%	139—35%
	1.0 "	52—13 "	47—12 "	246—62 "
	10.0 "	178—45 "	105—26 "	354—89 "
Brooklyn supply (933 samples)	0.1 "	26— 3 "	82— 9 "	285—31 "
	1.0 "	119—13 "	221—24 "	642—69 "
	10.0 "	418—45 "	408—44 "	868—93 "
Totals (1,332 samples)	0.1 "	42— 3 "	102— 8 "	424—32 "
	1.0 "	171—13 "	268—20 "	888—67 "
	10.0 "	506—45 "	513—39 "	1,222—92 "

broth the formula was adhered to as a test for B. coli. There are also included in the table the tests in dextrose broth showing 5 per cent or more of gas, which gives a good idea of the number of gas formers present, the greater part of which are not B. coli, in surface waters of good sanitary quality.

Anomalies.[12]—The anomalies shown by the use of dextrose broth and the formula are well illustrated in the following table:

TABLE 2.

	Form of Anomaly			Manhattan Supply	Brooklyn Supply	Total
	0.1 c.c.	1.0 c.c.	10.0 c.c.			
Dextrose broth	+	o	o	10	26	36
	+	+	o	3	14	17
	+	o	+	4	20	24
	o	+	o	15	83	98
Total				32	143	175
Lactose bile	+	o	o	1	1	2
	+	+	o	0	0	0
	+	o	+	4	2	6
	o	+	o	6	7	13
Total				11	10	21

There is certainly much greater interference with proper gas production in dextrose broth than in lactose bile.

The second year's comparisons give a better idea of the true relative value of the test media, since transplanting to bile established very closely the actual amount of B. coli present. The following table gives the number and percentages of positive tests for B. coli obtained by the different media, and also in the last column the number and percentages of total gas formers present.

The first two columns show no very great difference between the lactose bile and the dextrose broth formula, there being a slight advantage for the bile.

The second and third columns illustrate the great improvement caused by adding peptone to the lactose bile.

The third, fourth, and fifth columns prove that the lactose bile with peptone produces in practice substantially the same results as rejuvenation in dextrose broth, made with Liebig's extract, combined with transplanting to bile. With Manhattan water the bile decidedly had the advantage.

The last column shows again that an excess of gas formers, not B. coli, may be present in water of good quality.

TABLE 3.

		Dextrose Broth (formula)	Lactose Bile	Lactose Bile with Peptone	Dextrose Broth, 5% Gas and Over, Transplanted to Bile	Duplicate Bile Tests	Dextrose Broth 5% Gas and Over
Manhattan supply (85 tests)	0.1 c.c. ...	0— 0.0%	1— 1.2%	1— 1.2%	0— 0.0%	2— 2.4%	0— 0.0%
	1.0 " ...	6— 7.1 "	5— 5.9 "	13—15.4 "	4— 4.7 "	15—17.7 "	14—16.5 "
	10.0 " ...	24—28.2 "	31—36.5 "	54—63.5 "	48—56.5 "	60—70.6 "	59—69.4 "
Brooklyn supply (160 tests)	0.1 " ...	1— 0.6 "	1— 0.6 "	1— 0.6 "	2— 1.3 "	2— 1.3 "	15— 9.4 "
	1.0 " ...	11— 6.9 "	15— 9.4 "	25—15.6 "	25—15.6 "	31—19.4 "	62—38.8 "
	10.0 " ...	51—31.9 "	63—39.4 "	72—45.0 "	93—58.1 "	88—55.0 "	121—75.6 "
Total (245 tests)	0.1 " ...	1— 0.4 "	2— 0.8 "	2— 0.8 "	2— 0.8 "	4— 1.7 "	15— 6.3 "
	1.0 " ...	17— 7.0 "	20— 8.2 "	38—15.5 "	29—12.0 "	46—18.8 "	76—31.4 "
	10.0 " ...	75—30.7 "	94—38.4 "	126—51.2 "	141—57.6 "	148—60.4 "	180—73.5 "

In the experiments conducted under artificial conditions the number of formula tests for B. coli was 58 per cent of the number of tests showing gas formation. In the above table it is interesting to note that the number of formula tests is 54 per cent of the number of tests for B. coli shown in dextrose broth by gas formation corroborated by transplanting to bile.

Rejuvenation tests.—About 60 rejuvenation tests were also made on these waters, planting in plain broth and transplanting to bile. The results are shown in the following table:

TABLE 4.

		Lactose Bile with Peptone	Plain Broth Transplanted to Bile
Manhattan supply (19 samples)	0.1 c.c.	1— 5.3%	0— 0.0%
	1.0 "	2—10.5 "	1— 5.3 "
	10.0 "	6—31.6 "	6—31.6 "
Brooklyn supply (38 samples)	0.1 "	1— 2.6 "	2— 5.3 "
	1.0 "	8—21.1 "	12—31.6 "
	10.0 "	27—71.1 "	32—84.2 "
Total (57 samples)	0.1 "	2— 3.5 "	2— 3.5 "
	10.0 "	10—17.6 "	13—22.8 "
	10.0 "	33—57.9 "	38—66.7 "

Rejuvenation in plain broth showed a slight advantage on the Brooklyn water, probably due to the fresh beef infusion in its composition.

The same samples were also used in a comparison between gas

formation in dextrose broth, made with Liebig's extract, before and after rejuvenation in plain broth with the following results:

TABLE 5.

		Dextrose Broth (Gas Formation)	Plain Broth Transplanted to Dextrose Broth
Manhattan supply (18 samples)	0.1 c.c.	9— 50%	13— 72%
	1.0 "	15— 83 "	15— 83 "
	10.0 "	17— 94 "	18—100 "
Brooklyn supply (37 samples)	0.1 "	7— 19 "	14— 38 "
	1.0 "	30— 81 "	31— 84 "
	10.0 "	37—100 "	37—100 "
Total (55 samples)	0.1 "	16— 29 "	27— 49 "
	1.0 "	45— 82 "	46— 84 "
	10.0 "	54— 98 "	55—100 "

There was considerable improvement after rejuvenation, showing the necessity for fresh beef infusion in dextrose broth, as previously indicated. An abundance of gas formers was again shown in water containing comparatively very little B. coli.

Transplanting.—Rather uncertain results may sometimes follow transplanting, so that as a routine procedure it is not to be highly recommended. This is due partly to interference of bacteria and largely to errors of inoculation.

The following table has been compiled from a large number of transplantings (360):

TABLE 6.

	B. coli Present	Gas in Dextrose Broth	Formula Correct	Transplanted to Bile	Number of Tests
A	+	+	+	+	40
B	+	+	o	+	72
C	+	o	o	+	9
					121
D	+	+	+	o	6
E	+	+	o	o	17
F	+	o	o	o	9
					32
G	o	+	+	o	21
H	o	+	o	o	99
					120
		Lactose Bile Positive Test			
I	+	+	..	+	15
J	+	o	..	+	11
K	+	+	..	o	6
L	o	o	..	o	55
					87

Among other things the table shows that a formula test in dextrose broth may be caused by other bacteria than B. coli in quite a percentage of cases (see G), and on the other hand that transplanting may sometimes fail to show B. coli altho present (see D, E, F, and K). Several times, transplanting to bile brought up a test when originally no gas had appeared (see C and J).

CONCLUSIONS.

1. Experiments conducted under carefully regulated conditions have shown that the bile salts in lactose bile cause an appreciable degree of inhibition in the development of B. coli.

2. This inhibition increases with attenuation.

3. Rejuvenation in suitable media followed by transplanting to lactose bile will sometimes prove the presence of B. coli, usually attenuated, not shown by the lactose bile in direct tests.

4. In actual practice covering hundreds of samples of Manhattan and Brooklyn waters, the lactose bile, made with the addition of 1 per cent peptone, has been shown to practically equal the results obtained by rejuvenation in dextrose broth, made with Liebig's extract, followed by transplanting to lactose bile.

5. If dextrose broth is continued in use, it should be made with fresh beef infusion, since it is then more delicate than when made with Liebig's extract. The formula test is of but little practical value and transplanting to lactose bile should be made as soon as gas appears.

6. Gas formation in lactose bile after transplanting from the plain broth or dextrose broth is not always certain, even when B. coli is present, on account of interfering growths in the original medium.

7. Lactose bile gives more reliable *presumptive* tests for B. coli than any other known medium,[16, 17, 18] including aesculin broth.[19, 20]

8. Other species of bacteria cause much less interference with gas formation of B. coli in lactose bile than in other media.

9. To rejuvenate and transplant seems too laborious and uncertain in routine work. The information gained assists more especially in interpreting the results obtained with lactose bile. Lactose bile makes a distinction between recent and distant contamination, hence gives better evidence of the actual relative sanitary quality of a water.

10. The use of lactose bile as a step in the separation of B. typhosus[21] from water adds yet weightier reasons for its direct employment in the examination of water.

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A HEMOPHILIC BACILLUS FOUND IN URINARY INFECTIONS.*

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WHILE a very great amount of work has been done on the bacteriology of urinary infections it appears that no systematic investigation, at least at all extensive, has been made with blood media. In a series of cases such media was used in the routine examination of the urine and other material from the urinary tract with the result that in three cases an organism was found which apparently can be cultivated only by this method. This organism is here described and an account given of the three cases from which it was obtained together with some data on its specificity.

Case 1. A man, 41 years of age, gave a history of turbid urine for at least 8 years and possibly for a much longer period. Thirty years ago he had some urinary trouble at which time he passed what was probably a small stone through the urethra. Since then he has had no symptoms suggesting calculi. Twenty-five years ago he had an attack of typhoid fever. At the present time he seems to have perfect general health. His nutrition is good and appetite excellent; he has no fever and has worked constantly for years as a salesman. There has always been some tendency to constipation but never marked. He has had no venereal disease. He is married and his wife and several small children are perfectly well. Urination appears to be normal. He is rarely obliged to get up at night to urinate and no symptoms of vesical irritation exist. Physical examination reveals nothing of importance. The left lobe of the prostate is slightly larger than the right; no secretion was obtained by massage. The kidneys are not tender or enlarged. X-ray pictures taken two years ago and at the present time show no evidence of calculi in the urinary tract. A cystoscopic examination made three years ago gave negative findings. Examinations of the urine for tubercle bacilli by the staining method and also by guinea-pig inoculations were without result.

Examination of the blood gave the following: Red cells 4,800,000; hemoglobin 90 per cent; the leukocytes were counted at about weekly intervals for several weeks and always ranged between 10,000 and 13,000. Differentiation gave an increase in the eosinophiles, the percentage of these cells varying from 6 per cent to 12 per cent; the other cells occurred in normal proportions. This eosinophilia led to careful examination of the urine and stools for parasites and ova but none have thus far been found. Possible echinococcus of the urinary tract was especially considered, but a history indicating the possibility of passing the parasite was not obtainable, and repeated examinations of the urine for hooklets, etc., have given negative results. The temperature taken morning and evening for two weeks was never above normal.

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The urine has been examined at frequent intervals for over a year. It was always obtained by having it passed directly into a sterile bottle after sponging the meatus and allowing the first urine to escape, and then immediately examined by smear and culture. It was always very turbid and on standing a voluminous white sediment soon appeared, the urine above becoming remarkably clear. The specific gravity was usually about 1,016 and a slight reaction for albumin and a distinct reaction for mucin could always be obtained. No sugar. The reaction was constantly alkaline to litmus but was never ammoniacal.

Microscopically no casts or red corpuscles were ever found. Polynuclear leukocytes were always present in large numbers and usually in a very good state of preservation. It was rare to find mononuclear or epithelial cells. Counts of the leukocytes in the urine made at intervals of one or two weeks for six months ranged from 1,200 to 4,000 per cu. mm. The bacilli which will be described later were always found in large numbers both within and without the leukocytes. Examinations have been made at short intervals for over a year and the case is still under observation. As a rule no other organisms have been seen in smear or culture. In two or three instances in smear preparations a few gram-positive diplococci were observed. They were not cultivated and were not constantly found. As these specimens were not obtained by catheter it is possible that they came from the urethra.

Case 2. This patient, a young woman, gave a history of having had, in April, 1908, an indefinite "grippe" infection which lasted four weeks and after which she did not properly regain her strength. Two months later she was exposed to cold and dampness and on the second day following, she noted stiffness in the left knee joint which soon became painful when she put her weight on it. One week later the other knee became similarly affected. Slight swelling occurred about the joint. She remained in bed for six weeks without perceptible improvement and during this time she also had pains in the elbows and finger joints which were not severe and of short duration. It was for this trouble that she entered the Presbyterian Hospital in the service of Dr. Billings in October, 1908. At this time no appreciable swelling of the joints was noted altho she complained of stiffness and pain on motion. The temperature occasionally rose to 99°; no chills. The red corpuscles numbered 4,846,000; the white corpuscles ranged from 10,000 to 15,000. At no time were definite urinary symptoms complained of. In a catheterized specimen of the urine, however, were found leukocytes and a small bacillus. The bacillus was cultivated and found to possess the cultural characteristics of the bacillus in Case 1. A vaccine was prepared and inoculations begun and continued weekly for nearly three months. Catheterized specimens of the urine were obtained each week for several weeks. They were always slightly turbid and contained a few polynuclear and epithelial cells but no red cells or casts. The leukocytes in number ranged from 20–50 per cu. mm. of urine. At times a small amount of serum albumin was found; and quite constantly there was a distinct reaction for nucleoalbumin; no sugar. The reaction to litmus as a rule was slightly alkaline. In every specimen there was found in smears and culture the small bacillus referred to; no other bacteria were present. They were much less numerous than in Case 1 but in the centrifuged sediment they were easily found, usually in small clumps about the leukocytes and the few epithelial cells commonly found. One drop of urine per plate gave in the first specimens examined from 20 to 40 colonies.

Case 3. This patient, a man 39 years of age, was in the service of Dr. Billings in the Presbyterian Hospital. He commenced to have spinal trouble about eight years

ago. He first noticed pain in the neck on turning the head and soon the pain extended downward involving the thoracic spine. Associated with the pain was spinal rigidity and deformity which advanced to a well-marked kyphosis. Three years ago he commenced to wear a brace which relieved the pain greatly and for the past two years he has had little or none and the deformity has not advanced. At the present time the entire spine is held rigid and motion in all directions is very much restricted. Slight crepitus is heard on rotation of the neck. Other joints of the body do not appear to be involved at present and have not been affected in the past. For many years he has had recurrent attacks of tonsilitis, two of which occurred before the spinal trouble began. These attacks are severe but of short duration. At present the tonsils are slightly enlarged and the surfaces irregular but are not acutely inflamed. The throat is normal. The tonsils were removed and in the crypts was a nearly pure culture of streptococcus

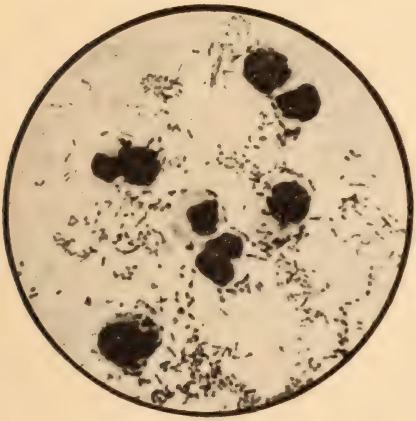

FIG. 1.—Hemophilic bacillus from urine. Smear preparation of urinary sediment from Case 1. ×1,000.

FIG. 2.—Hemophilic bacillus from urine. Pure culture three days from blood agar. ×1,000.

pyogenes. Thirteen years ago he had an attack of gonorrhea and has also in the past had attacks simulating asthma. Four years ago he had cystitis the nature of which was undetermined. At present his general health is good, and physical examination, excepting the findings given above, reveals nothing significant. The prostate is slightly enlarged and firm, and massage expresses a small amount of a thin discharge in which are leukocytes and intracellular gram-negative organisms suggestive of gonococci. They have not been cultivated. He complains of no urinary symptoms at present. The urine (catheterized) is clear, acid in reaction, contains no albumin, sugar, or casts, but an occasional leukocyte can be found. In blood agar plate cultures and in smears of the sediment were found a considerable number of small gram-negative bacilli which grow only on blood media and in every respect correspond to the organism from Cases 1 and 2. No other bacteria were found.

Morphology.—The organism is a small non-motile, gram-negative bacillus whose size varies considerably but is usually about that of the influenza bacillus. At times, and especially in the urine, it is distinctly larger and may be quite plump and short. Its measurements may be given at from 0.2–0.3 μ.×0.5–0.8 μ. It stains readily with ordinary dyes and is non-acid and non-alcohol fast. With methylene blue it

often stains unevenly, sometimes more deeply at the ends, and again fairly distinct granules may be made out so that it looks not unlike very minute diphtheria bacilli. There is no tendency to form threads or chains, and a characteristic grouping has not been made out tho it is common to see the organisms both from urine and from cultures arranged in small irregular clumps consisting of several bacilli. In the urinary sediment they may be seen clustered about the pus cells, probably having been thus carried in the process of sedimentation. They occur both within and without the leukocytes and at times seem to adhere to epithelial cells as was observed especially in Case 2. The bacillus does not form spores and does not possess a capsule.

Cultural Characteristics.—The rather remarkable cultural characteristics of this organism first attracted my attention. In Case 1 the large number of small bacilli were noted in the smear preparations, and when aerobic blood plates were made no growth appeared. From a second specimen similar plates were made and placed under aerobic and anaerobic conditions. In the latter in 24 hours definite small colonies were seen, evident to the naked eye chiefly on account of the clear, wide zone of hemolysis surrounding them. In this instance, too, much smaller tho similar colonies were seen on the aerobic plates after 24 hours. As a rule the bacillus grows more luxuriantly and more quickly under anaerobic conditions and failure to grow under aerobic conditions is occasionally noted on blood plates. Growth always takes place slowly and continues for several days. This is more noticeable on blood agar slants where often no growth is evident to the naked eye in 24 hours. At the end of 48 hours, as a rule, very delicate growth is manifest in the form of tiny, discreet, dark grey colonies. It continues to increase slowly until the fourth or fifth day, after which no further growth is apparent. It never becomes profuse and may require the aid of a hand lens to be seen at all. It is slightly raised above the surface, is easily removed with the loop, and is not tenacious or slimy. This organism does not grow at room temperature. On the plates the colonies as a rule in 24 hours are grey, almost invisible to the eye, and the clear zone of hemolysis measures from 0.5 to 1 mm. in diameter. There is no tendency for the growth to spread on the surface. Under the microscope the colonies are finely granular, grey or greyish brown, the margins are regular and about the colonies the red blood corpuscles have entirely disappeared. The outer margin of the zone of hemolysis is usually quite definite, being in this respect similar to the zone about streptococcus colonies, altho often it may be seen to shade more gradually into the surrounding media. The zone of hemolysis is not colored but often is slightly hazy. The colonies present the same appearance on plates with human, rabbit, goat, and pigeon blood. Attempts to grow this bacillus on media not containing blood have persistently failed. Plain agar, glycerin agar, ascites agar, Löffler's blood serum, lecithin agar, potato, gelatin, broth, milk, and sugar media give no growth under either aerobic or anaerobic conditions. When a few drops of blood, either human or animal, are added, growth occurs. Fluid blood media are not as favorable as solid blood media. Milk and various sugar broths to which blood has been added show no change or fermentation. Attempts to grow it in alkaline and acid normal urine pure or when added to agar or broth have failed. This was somewhat surprising in view of the fact that the bacilli in the body develop in urine which apparently is free from blood. Filtered patient's urine and filtered nephritic urine give no growth. Plain media made so as to contain varying percentages of acid and alkali likewise gave negative results. In Libornius tubes good growth was visible in blood media but never obtained when ascites, sugar, or other

media not containing blood were used. Strains of the bacilli from the three cases have been cultivated side by side on the various media and no noteworthy differences have been observed at any time. The organisms from Cases 1 and 2 have been grown on blood agar slants now for several months, transfers having been made every four or five days or oftener, and the growth is the same in appearance and the bacilli have the same morphology as when first isolated. Growth is possibly slightly more profuse after long cultivation and the bacillus shows no tendency to die out as was observed when first isolated. Several times in recently isolated cultures growth in subculture was not obtained after three or four days. As a rule the viability is much greater. In one blood agar tube, kept moist and at room temperature, the bacilli grew at the end of 12 days, 18 days, 23 days, but not after 30 days. In the patient's urine (Case 1) they die much more quickly. A specimen of urine was kept in the ice-box, plate cultures being made from day to day with definite amounts of the urine. Plates inoculated with four drops of the urine gave approximately 1,200 colonies immediately after voiding; after 24 hours approximately the same number; after 48 hours there was a decrease to about 800 per plate; after 72 hours about 100 colonies per plate; after 96 hours only a few colonies appeared on the plate and after 120 hours none were found. A similar experiment, leaving the urine at room temperature, gave approximately the same result. There was a gradual decrease in the number of colonies each day and at the end of five days the urine was sterile. Heating to 55° C. for 10 minutes suffices to kill this organism when suspended in salt solution.

Pathogenicity.—For animals the bacillus has little or no pathogenic power. Inoculated subcutaneously abscess formation does not occur in rabbits or guinea-pigs. Intraperitoneal injections in very large doses may cause death in rabbits in 24 hours and the organism may be recovered from the peritoneal fluid. They have not been recovered in the heart's blood or in other fluids. Intravenous injections in rabbits gave no result.

Phagocytosis.—In the urine there is always a considerable amount of phagocytosis apparent tho the amount may be variable. At times 50 per cent of the leukocytes contain bacilli and again not more than 10 per cent contain them. It is common to see the bacilli clustered about the leukocytes as if adhering to the surface. In vitro they are readily phagocytable in serum and to a certain degree spontaneously. In Case 1 opsonic indices were taken at about weekly intervals for two months and during this time injections of the dead bacilli were given. Beginning on June 19 the opsonic indices were as follows: June 19, 1.6, June 24, 4.5, July 6, 6.4, July 19, 4.9, July 21 3.0, July 29, 2.6, August 8, 1.6, August 25, 2.8. On June 24 inoculations with the dead bacilli were begun and continued about every 10 days for three months in doses of about 150 million at first and gradually increased to one billion. Marked local reaction occurred with each injection and after the larger doses some general reaction manifested by slight rise in temperature, feeling of depression, some muscle soreness but no bladder irritability. The reactions were never severe. Examination of the urine from time to time during the period of inoculations and also after the injections were discontinued revealed no appreciable change with respect to the number of bacilli or to the amount of pus present.

Case 2 was also given injections with the dead bacilli each week for a period of eight weeks in doses of from 100 million to 600 million. Slight local tenderness always developed and as a rule there was slight rise in temperature and some increase in leukocytes in the blood but no vesical irritation. During this period the number of bacilli

in the urine decreased but they did not entirely disappear. The pus cells also became less, and in one or two specimens were absent, tho later they appeared again in small numbers. The patient complained less of the pain and stiffness in the joints tho probably little stress should be placed upon these subjective symptoms as she is of a neurotic temperament. Before the injections were given the opsonic index was 0.66. Later after several were given it was 1.8. Some spontaneous phagocytosis occurred.

Agglutination.—In Case 1 the serum was tested for the presence of agglutinins, at six different times, twice before the inoculations were begun and again later. Tests were made both macroscopically and microscopically in normal and patient's serum and at no time was there distinct evidence of specific agglutination. In dilutions of 1:10, 1:20, and even 1:40 in both normal and patient's serum it was common to note microscopically clumping of the bacilli into small masses of 6 to 10 or more, but this was also seen in salt solution. The serum from Case 2 was also tested with negative results. A rabbit was injected 15 times about once a week with living cultures and from time to time the serum was tested for agglutinins but always with negative results. An opportunity was not afforded to test the serum in Case 3.

Bacteriolysis.—Suspended in normal and patient's serum using various dilutions with salt solution this bacillus shows no evidence of lysis either microscopically or by the plate method. After 18 hours there was a slight reduction in the number of colonies on the plates which was about the same in normal and in the patient's serums unheated and heated. No increase in the number of colonies takes place when the suspensions are incubated. Rabbit's serum in small amounts failed to show any evidence of reactivation of heated (56° 30 min.) patient's (Case 1) and normal serum.

Complement Deviation.—In Table 1 are given the results of an experiment to determine the presence of specific antibodies in serum by the method of Bordet and Gengou. The results are those obtained after one hour's incubation. It will be noted

TABLE 1.

COMPLEMENT DEVIATION.

One Hour at 30° C.					Hemolytic System	Result Hemolysis
Mixtures	Serum 2 Drops	Complement (Guinea-Pig)	Antigen	Salt Solution		
1.....	Patient's Serum	.04 c.c.	2 drops dense living suspension	1 c.c.	Suspension red corp. 1 c.c. + 1/125 c.c. amboceptor	Trace
2.....	Normal "	"	"	"	"	Almost complete
3.....	Immune rabbit "	"	"	"	"	Trace
4.....	Normal rabbit "	"	"	"	"	Almost complete
5.....	Patient's "	"	....	"	"	" "
6.....	Normal "	"	....	"	"	" "
7.....	Immune rabbit "	"	....	"	"	Marked
8.....	Normal rabbit "	"	....	"	"	Almost complete
9.....	......	"	....	"	"	Complete
10.....	......	....	....	"	"	None

that with patient's serum (Case 1) and immune rabbit's serum (mixtures 1 and 3) the hemolysis was not completely inhibited, but compared with the normal sera (mixtures 2 and 4) greatly retarded. The experiment was made using varying amounts of serum (from 1 to 4 drops per tube) and also using varying amounts of antigen both living and

heat-killed. The results were similar in practically all the tubes except in the set of tubes containing the dilute antigen (1 drop of 1:3 and 1:5 dilution) in which case there was little or no evidence of inhibition of hemolysis. The living and dead antigen appear to act much alike, the dead antigen possibly being less favorable.[1]

Having observed this organism in the urine in three cases the question arises as to the frequency with which it occurs in pathologic conditions of the urinary tract and also as to whether or not it occurs at times normally. Since this bacillus was first found in Case 1, specimens from about 50 cases of urinary infections in men and women have been examined by the blood agar plate method with especial reference to this organism, and the two additional cases were found among this number. It might, therefore, seem that these bacteria may not be exceedingly rare if proper examinations are made to detect them. More examinations must be made to determine this, however. With a view of determining whether or not they occur in normal urine, catheterized specimens were obtained from women nearly all of whom were gynecological patients in the Central Free Dispensary of Chicago. Cases presenting evidence of infection of the urinary tract are not included in this group. In 43 cases[2] the urine was obtained under sterile precautions by catheter and immediately taken to the laboratory for examination. In all cases both aerobic and anaerobic blood agar plates using 1 c.c. of urine were made and examined from day to day for five days for bacteria. The urine was centrifuged also and the sediment examined for leukocytes, casts, etc., and in stained smears for bacteria. In 38 cases the plates remained sterile and nothing of importance was found in the urinary sediment. In the remaining cases bacteria were found in smears and culture but no pus cells or other urinary sediment. In two cases the colon bacillus occurred in pure growth but few in numbers. In one a few colon bacillus colonies appeared on the plates and in the sediment they were associated with a gram-negative coccus which did not grow in the cultures. In the remaining two were found gram-positive bacilli in small numbers which appeared on the plates, grew on ordinary media and belonged

[1] I am indebted for the hemolytic serum to Dr. W. E. Post, who at the time was using it for Wasserman tests which had been proved thoroughly trustworthy.

[2] These cases were women who came to the dispensary for treatment and include chiefly diseases of the uterus and its appendages. I am indebted to Dr. Heaney and Dr. Rasmussen for aid in obtaining the specimens.

to the pseudo-diphtheria group. In no instance did I find a bacillus similar to the organism now described.

Concerning the significance of this organism in urinary infections definite statements at the present time perhaps may not be made. It is probable that like the colon bacillus it may find suitable conditions for its development produced by other primary infections or by mechanical processes. It evidently is not highly pathogenic.

The literature on the bacteriology of urinary infections is very extensive and the most important works have been consulted carefully to determine whether an organism corresponding to the one I have described has been observed before. As previously stated nearly all of the cultures have been made with non-hemoglobin media, which are quite worthless in isolating this organism. This is true, for instance, of the extensive work of Rovsing, Melchior, Faltin, Brown, Raskai, Suter, Tanaka, and others, which includes many hundreds of cases. There is an occasional reference to cases in which organisms were found in smears in the urine but were not cultivable. Scant attention has been given to these and it is not unlikely that some of these cases may have been infections with this hemophilic bacillus.

The fact that this bacillus appears to require hemoglobin for its continued growth suggests at once its relationship to Pfeiffer's bacillus (B. influenzae). While in this respect it is similar, in other features it is so strikingly different that there is little reason for confusing the two organisms if proper tests are made. Briefly the differential points are as follows: My bacillus is always hemolytic on blood agar plates; the influenza bacillus does not hemolyse. The influenza bacillus shows the phenomenon of symbiosis; this bacillus does not. The influenza bacillus grows rapidly, requires only a very minute amount of hemoglobin and shows a distinct preference for pigeon's blood. This bacillus grows slowly, requires relatively large amounts of blood in the media and shows no preference for pigeon's blood. The colonies of influenza bacillus as a rule are distinctly larger and are more transparent and "dew drop" like than are the colonies of this bacillus, which are very minute and more opaque. Morphologically the influenza bacillus is more uniform in size and outline and in older cultures tends to produce thread forms, which this bacillus never does.

In the literature are a few cases in which Pfeiffer's bacillus is reported to have been found in the urinary tract and the question arises as to the possibility of the organisms from some of these cases having been confused with the bacillus here described. Cohn,[1] in a case of acute urethritis, found in smears and culture of the purulent discharge a bacillus in mixt growth with another encapsulated bacillus which Neisser identified as B. influenzae. It grew only on hemoglobin media, preferred pigeon blood and in other respects corresponded with Pfeiffer's bacillus. Later cystitis developed but no statement is made whether or not the bacillus was found in urine from the bladder. J. H. Wright[2] found in mixt culture in a case of pyelonephritis associated with a stone a bacillus which culturally and morphologically agreed with B. influenzae. Paltauf and Kretz[3] found the influenza bacillus in the kidney post mortem. From the descriptions given in these reports there is probably little doubt but that the organisms were B. influenzae. At any rate they are distinctly different from the bacillus here considered. It is of interest to note that we may find in the urinary tract at least two kinds of hemophilic bacilli which without careful differentiation may be confused. Friedberger[4] found in the preputial secretion of dogs a bacillus- "B. hemoglobinophilus canis" which resembles this bacillus in some respects more closely than does B. influenzae. It is a gram-negative, non-motile, small bacillus requiring hemoglobin for growth, and like our bacillus does not manifest symbiosis. However, it is differentiated by its failure to grow under anaerobic conditions, its uniform staining, and by the fact that it attains its maximum growth in 24 to 30 hours. Wolff[5] described an organism isolated from a rat which was hemophilic and gram-negative but somewhat larger than the influenza bacillus. After three months' cultivation on artificial media it acquired the property of growing on non-hemoglobin media. Our bacillus after four months cultivation has shown no such tendency.

Attention has been called to the fact that the bacillus at times bears some resemblance to forms of B. diphtheriae and its possible

[1] *Deutsche med. Wchnschr.*, 1905, 31, p. 1152.

[2] *Boston Med. and Surg. Jour.*, 1905, 152, p. 496.

[3] Quoted in *Modern Medicine*, 2, p. 482.

[4] *Centralbl. f. Bakt.*, 1, 1903, Orig., 33, p. 401.

[5] *Ibid.*, 1, 1903, Orig. 33, p. 407.

relationship to this group should be thought of. Pfeiffer[1] in an extensive article on the bacteriology of the urethra found pseudodiphtheria bacilli in 83.7 per cent of the cases examined. He divides the strains isolated into two groups, one in which growth is scant and a second in which growth is much more luxuriant. The bacilli of the first group remind one of our bacillus inasmuch as he states that at times on plain agar it does not grow, but grows well, tho slowly, on serum agar, which media he used to isolate it. However, the bacillus is much larger, being 0.3 μ to 0.8 μ wide and 2 μ to 3 μ long, is gram-positive, grows on non-hemoglobin media, and grows well on milk, which it acidifies. His second group corresponds to the more common pseudodiphtheria bacillus, which has little resemblance to our bacillus. Pfeiffer, however, isolated a small bacillus in one case which may very well be identical with our organism. It was a minute, gram-negative bacillus very similar, he states, to B. influenzae, and its colonies were small and finely granular. It grew on his beef serum medium but not on plain media and died after the third generation. Inasmuch as serum practically always contains some hemoglobin its growth may thus be explained. His description is very brief and he refers to it as an influenza-like bacillus. It is of interest also that in the literature occasionally one meets with statements that influenza-like bacilli were seen in smears of secretions of the urinary tract which did not appear in cultures. It is not unlikely that careful cultural work with blood media will reveal the nature of such bacteria.

SUMMARY

A bacillus has been isolated in pure culture from the urine in three cases. It grows only on media containing hemoglobin but differs in many important features from B. influenzae. Two of the cases had pyuria and the third gave a history of urinary infection some time previously. Two of the cases (Cases 2 and 3) had joint trouble but there is no evidence to indicate a causal relationship between the bacillus and the joint affection. There is reason to believe that this bacillus may not be rare and that it has been overlooked because of its hemophilic property.

[1] *Arch f. Derm. u. Syph.*, 1904, 69, p. 379.

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BALANTIDIUM COLI INFECTION IN MAN.*

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(From the Pathological Laboratory of the Tulane University of Louisiana, New Orleans, La.)

The infrequent occurrence of Balantidium coli in man has prompted the careful study of a fatal case, which occurred recently in the service of one of us (Dr. Bel), with a view of determining, if possible, the infectious nature of this infusorium. Interest in the case was stimulated by the fact that this is the first to be reported in an individual born in Louisiana who had never left the state, and the first to be carefully studied, both clinically and pathologically, in North America. We were fortunate to have seen the patient at an early stage of his complaint, and to have had the opportunity to follow, without interruption, the progress of the disease to its fatal termination. Moreover, as dysenteries are not uncommon in our semi-tropical southland, we are able to add another member, evidently endemic to this locality, to the already recognized bacillary and amebic types.

The significance of Balantidium coli in the intestine of man has been disputed by such workers as Malsmsten[1] and Doflein,[2] who hold to the harmless nature of the organism, and Strong,[3] Brooks,[4] Solowjew,[5] and others, who believe that the parasite is an important etiological factor in producing catarrhal and ulcerative lesions in the large intestine. From Strong's[3] and Solowjew's[5] studies in man, and the observations of Brooks[4] upon a fatal dysentery among the

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apes in the New York Zoological Garden, in which Balantidia were found, there is considerable evidence that these parasites are more than harmless commensals, and must be seriously considered as the cause of certain types of ulcerative colitis.

In perusing the literature on the subject, it is remarkable how little attention has been given to the lesions found in such cases—the authors seemingly devoting their studies to the biological features of the parasite, and the therapeutics, rather than attempting to solve the real pathogenic significance of the organism.

To Strong[3] and Solowjew[5] all credit is due, as the first to have carefully studied the lesions of the gut of fatal cases, and their interpretations and conclusions have thrown more light on the rôle played by these parasites than had been accomplished by recitals of clinical observations and therapeutical results. We have been greatly aided in our work by the excellent monographs of these authors.

While Strong[3] has been able to collect in the entire literature but 117 cases of balantidial invasion in man, of which but one is reported from the United States, we have personal communications from Dr. Mallory (Boston City Hospital) and Dr. Van Wart (Johns Hopkins Hospital) that a case had been encountered at each of these places in the routine study of autopsies, but, as these were incomplete, no attempt had been made to report them. Through the courtesy of Dr. Mallory, who sent us sections of the intestine from his case, we were able to compare the histological changes in it with those found in our case.

As invasions from this parasite have been reported from the cold climates of Finland and Russia, the temperate zones of North America, the tropical countries in South America, and the Philippine Islands, such wide geographical distribution precludes the possibility of associating infection by Balantidium coli solely with tropical or subtropical climates.

While the appearance of this infusorium in man suggests the possibility of its occurrence in other animals, it has as yet been found only in the intestine of swine, where it seems to be harbored without producing either marked clinical symptoms or pathological lesions.

Klein,[6] having found an organism which he believes to be Balantidium coli in sewage, and as the parasite has also been found in drinking water in London, suggests the possibility that infection may take place by this means. However, as 25 per cent of reported cases have occurred from direct contact with swine or following the ingestion of food products made from these beasts, little doubt exists as to the danger from this source. No better argument can be brought forward to confirm this belief than the instance related by Chichulin[7] in which an entire family had been infected after eating sausages made from a hog which harbored Balantidia. From the clinical history of our case, it appears very likely that infection was received through these animals.

CLINICAL HISTORY.

The patient, a colored male, age 40 years, was born in Louisiana and had never left the state. He was admitted to the Charity Hospital, June 22, 1909, complaining of diarrhea, abdominal pain, and tenesmus. His family history was negative. He had had the usual diseases of childhood, and pneumonia and gonorrhea later in life. Had had a sore on his penis 20 years before admission. Indulged freely in tobacco and alcoholics.

A railroad hand by occupation, he was always provided good drinking water, but kept pigs at home and was often called on to attend them. In October, 1908, he began suffering from diarrhea. His bowels would move from 8 to 20 times a day, the stools often containing blood. This condition persisted for six weeks, after which the stools became normal and remained so for a week, when the diarrhea returned. From this time to the date of his admission he had periodical attacks of diarrhea, the intermission between them growing less and his general weakened condition becoming worse.

On admission he complained of abdominal pain and severe tenesmus and of having 20 to 24 bowel movements each day. The stools contained blood and mucus and their character suggested those of amebic dysentery. Repeated examinations of the feces failed to show the presence of ameba or other protozoal or bacterial invasion, excepting Balantidium coli, which were always found in large numbers and very active.

Blood smears were negative for protozoa, anemias and leukemias—the total counts showing an average of:

Red blood cells	4,570,000
White blood cells	4,200
Hemoglobin	85%

The differential leukocytic count, taken at different times, averaged:

	Percentage
Polyneutrophiles	70
Large lymphocytes	2
Small lymphocytes	25.3
Large mononuclear	1.7
Eosinophiles	1.0

The examinations of the sputum and feces failed to show any acid-fast bacilli, altho the Morro cutaneous test gave a positive reaction.

The urine examinations were negative.

The temperature throughout his illness never exceeded 99° F. and was more often normal than otherwise. The pulse averaged 90, and the respiration 20 per minute. Blood pressure was 125 mm.

The physical condition of the patient was one of great emaciation—he reported having lost 70 pounds in weight during the 8 months previous to his admission. The skin was dry and the superficial lymph nodes of the neck, axillae, elbows, and groins were slightly enlarged. The pupils reacted normally to light and accommodation, and all other senses were normal.

Excepting a slight hebetude, the mental condition and nervous system generally were good.

The buccal mucous membrane showed a marked redness due to stomatitis, and the tongue was deeply furrowed and dry.

The thorax revealed pleuritic signs on the right side and the presence of many spots of consolidation in both lungs, which proved to be tuberculous. Shortly before death there were evidences of edema about the base of both lungs. The heart sounds were normal.

The abdomen showed marked retraction and a general absence of fat. There was tenderness on pressure, especially about the umbilicus and left inguinal region. The liver, spleen, and kidneys were negative, and, except for a scar upon the penis, nothing was noted abnormal about the genital organs.

The usual treatment for tropical dysenteries was administered, but, instead of improving, the patient gradually grew weaker and died, apparently from exhaustion, on September 7, 1909.

Briefly, were it not that Balantidium coli was found in the intestinal discharge, it would have been difficult to differentiate the intestinal upset in this case from that of an amebic infection—the intermittent diarrheal attacks, muco-bloody stools, tenesmus, and rapid emaciation of the patient were very suggestive of an amebic invasion.

Autopsy Protocol: 2871. Dave Williams.

Body is that of a poorly nourished, but well-developed, black male. Body length 179 cm. Pupils equal and measure 6 mm. Sclerae yellowish-brown in color. There is a scar on glans penis. The superficial lymph nodes are not palpable. Cadaveric rigidity is absent. The body is warm. Necropsy held 2 hours after death. The abdomen is moderately distended and tympanitic. The intestines are greatly distended with gas. The small intestine is smooth and glistening; the large intestine is lusterless, opaque, and feels boggy and thickened, especially about the sigmoid flexure and rectum. There are no adhesions, except a few fibrous tags about the spleen.

Lungs.—Both are adherent at the apex to the thoracic wall by old fibrous tags. Few nodular masses are felt below the pleura and deeper into both organs. On section, these are found to be well circumscribed tubercles; many containing calcareous deposits. Besides these, there are bright red discrete areas scattered throughout both lungs. These areas are not elevated, but remain on an even plane with the remaining portion of the organ. They are very irregular, suggesting "ink blots." The lower lobes of both lungs are edematous. The bronchial glands are pigmented and enlarged, and the bronchi are negative.

Gastro-intestinal tract.—The stomach is contracted and its rugae are prominent.

It contains about 200 c.c. of a greenish turbid fluid. Examination of scrapings from its wall are found free of Balantidia. The small intestine contains much sticky mucus throughout its length, especially abundant about the lower portion of the ileum. The villi are prominent and deeply injected, especially those on the crests of the valvulae conniventes. No ulcerations or muco-purulent exudate can be found. Scrapings from the ileum, about 20 to 30 cm. from the ileocecal valve, show, on examination, a few living Balantidium coli. Similar scrapings from other portions of the ileum and jejunum are negative to Balantidia. The large intestine is swollen, thickened, and edematous throughout its length; more so at the curves, and especially so at the sigmoid flexure and rectum. The thickening does not appear to be due to an increase of connective tissue, but, rather, to edema. It seems to be confined to the mucosa and submucous coat. The mucosa is studded with shallow ulcers; their contents are muco-purulent and not adherent to underlying tissues. There is no diphtheritic membrane present. The ulcers are largest and most numerous in the sigmoid flexure and rectum and especially in the latter structure. There are also many smaller and more discrete ulcers at the different curves, probably for the same reasons that induce their presence at these points in other intestinal infections. The ulcers are not undermined nor heaped, but rather shallow with tapering edges. In general the gross appearance of the large gut does not suggest amebic dysentery. Examination of the exudate from the structure shows many living Balantidia. They are especially numerous where the lesions are the most marked at the angles of the gut and in the sigmoid and rectum.

Brain.—The ventricles are distended with a clear fluid. Two bright red clots, originating from the choroid plexus and adherent to it by a thin pedicle, extend forward into both lateral ventricles, and rest on the floor of the ventricles, without, however, being attached to their walls, but floating in the excess of contained fluid. On the velum are also noticed a few red points.

All other organs are normal.

Anatomical Diagonsis.—Chronic Pulmonary Tuberculosis (quiescent); Acute Ulcerative Colitis; Chronic Pleuritis; Intraventricular Hemorrhage (cerebral).

Aside from the slight lesions found in the lungs, there were no other evidences of tuberculosis to account for the marked cachetic condition of the patient; the rapid emaciation, intermittent dysentery, with tenesmus and bloody stools, suggested the picture of tropical dysentery. The failure to find ameba, both ante-mortem and at necropsy, eliminated the possibility of infection by these organisms, and the macroscopical appearance of the gut, with its small shallow ulcers, suggested a bacillary rather than a protozoal invasion. Its true nature was readily determined by the histological study.

Microscopic Anatomy.

With the exception of small petechial hemorrhages in various organs, and the pulmonary lesions, nothing is found on microscopic examination of sections from the lungs, heart, liver, spleen, kidney, pancreas, brain, or stomach, to suggest invasion by Balantidium coli. The mesenteric lymph nodes are enlarged, and there is a marked increase in their lymphoid cells. No Balantidium coli found.

The small intestine is normal, except about the lower portion of the ileum where a slight derangement of the epithelial cells, a moderate amount of mucus containing some polymorphonuclear leukocytes, and a marked injection of the blood vessels offer the only evidences of inflammation. Balantidia cannot be found, either in

the glands or in the exudate, altho they were present in scrapings of the ileum at autopsy.

The mucosa of the large intestine, aside from definite ulcers, is uniformly infiltrated with inflammatory cells. The ulcerated sites show an absence of glands and are filled with polymorphonuclear leukocytes, many lymphoid and plasma cells, and a moderate number of eosinophiles. There is little or no fibrin present. These ulcerated areas do not seem to extend much below the muscularis mucosa, the greater number appearing to be limited by it (Plate 3, Fig. 4). Some of the more recent ulcers are confined to a single Lieberkühn gland; the inflammatory process having followed downward from the orifice of such gland, the epithelial lining is lost for the most part. Capping the ulcers, and extending for a considerable distance over the edges, is a dense layer of polymorphonuclear leukocytes held in a delicate reticulum of fibrin. Balantidium coli is often found in large numbers in this exudate. The absence of acute inflammatory exudate in the submucosa is evidence that the superficial process is secondary. In those parts of the mucosa where ulcers are absent, the interglandular supporting tissue is densely infiltrated with lymphoid and plasma cells, the latter predominating. Altho a few eosinophiles are present, there is an absence of polymorphonuclear leukocytes. The small blood vessels in these locations are deeply injected, and some show a slight perivascular leukocytic zone. The conditions are responsible for the swollen appearance of the mucosa in the gross. The glands are, in places, greatly compressed, many showing complete obliteration of their lumen; in other parts they are dilated and cystic, and lined with a single layer of compressed epithelium (Plate 3, Fig. 4). These conditions of the glands have resulted from the interglandular cellular infiltration. Balantidia are found in the lumen of the glands (Plate 4, Fig. 1) and also superficially and deep into the interglandular supporting tissue (Plate 4, Figs. 2 and 3). The lesions in these areas may be regarded as subacute or chronic, and evidently incited by the presence of the organisms.

In the submucosa the connective tissue is pushed apart, the result of serous exudate (edema), and apparently by the burrowing of Balantidium coli through the tissues. The perivascular lymph spaces are filled with lymphoid and plasma cells; and similar cells are scattered throughout the submucosa. The blood vessels are deeply injected; especially is this marked in the region of the mucosal ulcers. There is no proliferation of their endothelial wall. Some of the vessels contain Balantidium coli (Plate 4, Figs. 4 and 6). The lymphatics are swollen but show no activity on the part of their endothelial lining. The solitary follicles show no apparent histological changes. Some of the nerve plexuses in the vicinity of the ulcers show a marked polymorphonuclear leukocytic invasion obliterating the plexuses almost entirely. There is an absence of phagocytic cells of any sort. The muscularis mucosae throughout some of its extent is disarranged, swollen, and edematous.

In attempting to interpret these findings, it was noticed that the small shallow ulcers were mostly limited to the mucosa, only a very few penetrating the muscularis mucosae, and none extending any distance into the submucous coat. From the exudate it was evident that the condition about these ulcers was mostly acute in

character, and that while Balantidium coli was found in the loose exudate that capped the ulcers, none was found in their walls where the lesions were active. On the other hand, at some distance from the ulcers, in the lumen of the glands, which were but little affected, in the thickened interglandular supporting tissue, and in the submucous coat, Balantidium coli was found in great numbers. As both the interglandular tissue and the submucosa were infiltrated with cells, characteristic of subacute or chronic inflammation, and as the parasites were present in, or very near to, these lesions, it can be inferred that they were responsible for its existence. The presence of the parasite in the blood vessels and lymph channels in the submucosa argues well for the possibility of carrying the infection to the liver or lung through these routes, and, while we could not find Balantidium coli in either organ, the hemorrhagic areas found in the lungs are so unusual and so suggestive as to warrant the suspicion that the parasites were directly or indirectly responsible for their existence.

In a study of the intestinal flora, at three different periods of the patient's illness, and also at autopsy, none but the usual bacteria common in the intestine could be found, altho a diligent search was made especially for members of the typhoid-dysentery group. Moreover, frequent blood examinations for the agglutination reactions against typhoid, paratyphoid, and the dysentery group, and blood cultures, both antemortem and postmortem, failed to reveal specific agglutinins for these bacteria, or the presence of an organism which might have been responsible for the existing condition.

BIOLOGY.

The parasite studied by us belongs to the ciliated infusoria; is egg-shaped, and measures from 0.07 to 0.1 mm. in length, by 0.05 to 0.07 mm. in breadth, with a funnel-shaped mouth situated on its ventral portion, a little below the anterior or more pointed pole. The peristome is beset with a thick layer of long cilia; their motion, being in a circular direction, suggests the action of a rotary lawn sprinkler. By these cilia, the organism is able to grasp its food, consisting of various cells and bacteria, from the feces. The

funnel-shaped mouth communicates with the endosarc through a short gullet, this latter structure extending backward and downward.

The endosarc consists of coarse and fine and highly refractile granules, inclosed in a thin and very transparent sheath, continuous from the gullet above and ending at the anus situated at the posterior or more rounded pole. This observation does not conform with Strong's[3] assertion that this granular matter of the endosarc is surrounded by a layer of spirally striated protoplasm. We have been able, by very simple means, to prove this point beyond doubt. One drop of a weak aq. sol. of eosin was placed on the side of an ordinary slide preparation of feces containing Balantidia. The stain, quickly diffusing through the feces, was refused by the organisms which were feeding. In a short while the sheath of the endosarc would be seen to contract from downward upward, carrying before it its contents, until this appeared as a granular spherical body immediately behind its mouth; the lower portion of the sheath, still attached to the anus, remaining as a broad band (Plate 1, Figs. 13 and 14).

A nucleus, usually kidney-shaped, lies in the endosarc below and, usually, to one side of the peristome. It is quite movable, changing its position constantly with the motion of the granular material. Its protoplasm, in the young and adult parasites, stains intensely and homogeneously by ordinary stains; but becomes granular and reticulated when segmentation is about to take place.

Vacuoles in the endosarc have been seldom seen by us in the parasite while feeding, and never while segmenting; but we have repeatedly observed them when the organism was at rest after feeding. Their constant change in number and size, together with a churning motion of the protoplasm and destruction of cells ingested, suggested the probability that their function is concerned with metabolism. When present, they number from one to six in a single parasite. While these vacuoles appear and disappear and fuse with one another and separate again, it cannot be said that they are contractile, for, while they may disappear from the field, by carefully focusing they can be followed through other parts of the endosarc. However, as Stein[16] has pointed out, they are connected by lacunae. In this manner, several smaller ones may form larger

ones, until, at times, the whole lower third of the parasite is taken up by a single large vacuole. We have often seen parts of larger, or entire small vacuoles, expelled through the anus, surrounded by a thin rim of protoplasm (Plate 1, Figs. 6 and 7); these, after a short time, would burst and become lost as granular matter in the surrounding medium.

Inclosing the endosarc, and separated from it by a narrow rim of homogeneous protoplasm (the ectosarc), is a thick cuticle striated longitudinally, the striations extending from the peristome on the ventral surface, and from a point on a level with it on the dorsal surface to the anus at the posterior pole. These striations are undoubtedly muscular structures and, as each is beset with a row of cilia extending throughout its entire length, it is evident that they control the movements of these, as well as the various motions of the parasite.

Cilia are to be found around the peristome, where they are longest, and around the longitudinal striation of the cuticle; there are no cilia between these bands and only a few irregularly scattered ones on the end of the anterior pole above the beginning of the striations.

By the aid of the coarse cilia and muscular bands, the parasite was able to move rapidly through fecal matter, and the comparative ease with which it is capable of moving large particles of matter which happen in its path attests the force of this motion. By contracting these muscular bands, the organism may become perfectly round and quite often uses this means in changing its route. Again, the parasite may become quite elongated or spindle-shaped when it attempts to pass between objects too large to be moved (Plate 1, Figs. 4 and 5).

Pseudopods, formed by protrusions of the endo- and ectosarc, have been encountered in a few instances; they are not limited to any part of the parasite, but may appear to either side, or about the posterior pole. They are more often about the latter site. They may be single, or many may be present simultaneously. We believe them to be a beginning of degeneration, for, after becoming spherical and attached to the mother cell by a thin pedicle, they are cast off as granular masses, and disintegrate.

Gurvich[8] and Strong[3] believe that Balantidia die by discharging the greater part of their granular endosarc through the mouth; the latter author also reports that he has seen this granular material discharged through the anus and through the breaks following the rupture of pseudopods. We have never observed the former phenomenon, but have often witnessed and produced by artificial means (weak solutions of sodium hydroxide or quinine, applied to the edge of the cover glass) the spontaneous rupture and discharge of the granular mass of the endosarc through the latter openings (Plate 1, Fig. 15).

While the influence of the warm stage plays an important part in accelerating their motion and hastening segmentation, we were unable to prolong the lives of the parasites either by incubation or by room or refrigerator temperature; disintegration and death seemed to be due more likely to chemical changes in the feces than to any thermal cause (Plate 1, Figs. 11 and 12).

Conjugation.—Wising[9] has described a method of conjugation by which two individuals become united at their peristome, fertilization taking place by the interchange of plasma. Woit[10] and Gurvich,[8] on the other hand, have described what they believe to be conjugation and reproduction, in which two individuals become encysted together with subsequent segmentation into spherules.

We have repeatedly observed a phenomenon in our parasite, which, from its constant occurrence when segmentation is about to take place, suggests itself as the most plausible description of conjugation.

Two adult parasites come together and at once begin to rotate around each other, but in an opposite direction. When their anal extremities meet, their motion slows for an instant and both press down upon the other. Their motion is again accelerated, rotation continues until these parts meet again, and the process is repeated. This continues until segmentation is well advanced. Coarse granular bodies are being constantly discharged from the anal orifices of both individuals during conjugation; these, being pushed aside by the rotary motion of both parasites, form around them a nestlike arrangement in which they remain until segmentation is complete. While we believe that an interchange of these granules may pass

from one parasite to the other during conjugation, we were unable to confirm our belief. We have observed the above phenomena for several hours, with a high dry objective, never having to move the slide to keep the two individuals in the field (Plate 2, Figs. 1, 2, 3).

Reproduction.—Many methods of reproduction have been described by various authors; thus, Gurvich[8] believes that he saw segmentation into spherules following conjugation of two individuals in one cyst; others have described the process by budding, the parasite revolves on its axis several times, the cilia stop, and a protrusion of ectosarc occurs. This increases in size, becomes globular, acquires nuclear granules and granular particles from the endosarc of the mother cells, the pedicle connecting it breaks, and the young parasite becomes free. Bushuyeff[11] observed ameboid movements in these segments. Lavrovskaya[12] observed the process of separation from the mother cell; she also observed granular bodies surrounded by a membrane inclosing a nucleus and vacuoles, but failed to find cilia or motion to these bodies. Bushuyeff[11] also observed these forms without motion or cilia, and Zhegaloff[13] saw movement in their granulation. These authors were, however, unable to witness the transition of these protoplasmic bodies into adult parasites. We have often seen such bodies with and without vacuoles, formed from motile as well as resting parasites, having watched their development and their subsequent separation from the parent organism; but we have always seen them disintegrate. They must be regarded as degenerations, as the death of the parasite from which they originated has invariably followed (Plate 1, Figs. 6, 7, 8, 9, 10). Gurvich[8] did not observe budding, but found moving bodies half the size of adult Balantidia which were supplied with cilia. Strong[3] suggests that the bodies described by Gurvich[8] were evidently young parasites. We are able to confirm Strong's[3] statement, as we frequently met such bodies with poorly defined nucleus and peristome, with slowly moving cilia, barely more than one-third the size of an adult Balantidium; the slow motion of these young parasites being the result of recent segmentation (Plate 3, Fig. 1).

We believe that reproduction takes place only by amitotic divi-

sion, as Ekecrantz,[14] Wising,[9] Leuckart,[15] Strong,[3] and others contend. The process, as we have seen it repeatedly, is as follows: After rotation and conjugation, as described above, has continued for some time, the outer cuticle of both parasites becomes lax. As segmentation of one parasite usually precedes by some time that of the other, the cuticle of the one nearest division is the more lax and consequently overlaps that of its mate. Their rotary motion is now sluggish and their nuclei become reticulated and elongated. About this time there appears about the center of the parasite a ciliated girdle which, slowly contracting, shows plainly a constriction, at first shallow, soon becoming deeper, and carrying with it the endosarc. Division of the nucleus takes place, each segment receiving its portion. The two segments are now connected only by a thin pedicle consisting of the outer cuticle and sheath of the endosarc; the pedicle breaks and complete division follows, the band of cilia around the parasite becoming the adoral cilia of the lower segment, while at the point of separation of the upper segment is formed its anus. The cilia movements of the lower stop shortly before complete division takes place, to recur only after the peristome and nucleus are well developed. These young parasites, hardly motile, with an ill-defined peristome and granular nucleus, correspond to those described by Gurvich as transitional forms (Plate 2, Fig. 1).

Encysted Forms.—Leuckart[15] and Stein[16] have described encysted Balantidia in which the parasite loses its cilia and becomes rounded, its endosarc contracts and contains fat globules, the nucleus is obscured, and the whole mass is surrounded by a thick capsule. We have observed these, excepting that the nucleus always appeared to us quite distinct, and the outer capsule, while well defined, did not appear thicker than is usually found in the active forms. It is possible that the forms observed were ones not completely encysted (Plate 3, Fig. 2).

Cultivation.—Our attempt to cultivate the parasite on Novy-MacNeal's media using human and rabbit blood on acid and alkaline agar, on Musgrave and Clegg's media with different degrees of acidity and alkalinity, in acid and alkaline broth and on water-agar titrated from 1.0 acid to 2.0 alkaline, using the feces and its

bacteria as symbiotics, has met with little success. Results might have been different had not the unexpected termination of our case forbidden further attempt.

Animal Experiment.—Wising,[9] Rapshevski,[17] Chigayeff,[18] Lavrovskaya,[12] Zhegaloff,[13] and Strong[3] have injected feces containing Balantidia in cats, dogs, rabbits, pigs, and monkeys with and without previously traumatizing the parts, with negative results. Moreover, Grassi[19] and Calanruccio[20] were unable to infect themselves after injecting Balantidia from the hog. On the other hand, Vlayeff[21] produced lesions in two cats by injecting in the stomach of one and in the rectum of the other feces containing the parasites; and Cassagrandi and Barbagello[22] and Chichulin[7] produced lesions in these animals after colitis had been induced by trauma. These observers, however, failed to find, or to record, the presence of Balantidia at their autopsies on these animals, and no mention is made of the study of symbiotics injected with the feces. It is possible that the catarrhal condition of the bowels found might have been due to the bacteria contained in the feces with the parasites, or to the entrance of bacteria already present in the traumatized gut, rather than to the parasites which were evidently not recovered at autopsy.

From the conflicting results obtained by these observers it was evident that no definite conclusions could be arrived at by repeating their experiments unless artificial cultivation had been successful and the organism obtained in pure culture. As we have failed to accomplish this, we believe that a correct understanding of the rôle played by these parasites can be obtained far better by a careful study of the proper interpretations of the histological findings, than by experiments with an organism which can only be fed or injected to animals in symbiosis with pathogenic or non-pathogenic bacteria.

SUMMARY AND CONCLUSIONS.

We have repeatedly found Balantidium coli in large numbers in the stools of patients during life; we have found them active and very numerous about the lesions at autopsy. In the intestines of both Dr. Mallory's case and ours, we have found Balantidium coli in sections of the glands of the large intestine and interglandular

supporting tissue, the submucous coat, and in the blood vessels, and, wherever the parasites were present, lymphoid and plasma cells and eosinophiles were constantly in evidence, whereas the absence of such cellular infiltration foretold a negative finding of the parasite.

We believe that ulcerations are due to terminal invading bacteria, as evidenced by their acute character (polymorphonuclear leukocytes, fibrin), and while we do not doubt that Balantidium coli is primarily responsible for their presence by producing avenues for the entrance of these bacteria, the absence of parasites from the walls of these ulcers is sufficient evidence that they play no further part in their production.

From our study of the intestinal flora and the negative blood reactions for specific agglutinins, we are satisfied that any of the bacteria normally present in the intestine of man may produce the ulcerations after Balantidium coli has opened the avenues for infection. We believe that Balantidia produce these definite lesions (hyperplasia and cell infiltration) either mechanically or through the liberation of cytolytic ferments.

The presence of the parasite in blood vessels and lymph spaces leaves no doubt that infection of the liver and lung may occur through these channels in a manner similar to that described by Gage[23] in an invasion of the lungs by Strongyloides intestinalis, or as frequently happens in intestinal amebiasis.

Finally, from the definite and constant microscopic findings and negative blood and cultural results for other intestinal invaders, the logical conclusion seems to be that Balantidium coli is not a harmless commensal, as some authors suppose, but an organism able to invade the human tissues and cause a serious disease; death may follow through compression of the intestinal glands by a hyperplasia of interglandular tissue produced by the parasites and through glandular necrosis and absorption of toxins from any terminal bacterial invasion.

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21. VLAYEFF. *Vratch*, St. Petersburg, 1898, 19, p. 140.
22. CASSAGRANDI AND BARBAGALLO. (Quoted by Strong.)
23. GAGE. *Jour. Med. Res.*, 1910, 23, p. 177.

EXPLANATION OF PLATES.

PLATE 1.

FIGS. 1–5.—Active stage of the organism, Fig. 4 representing the form assumed by a parasite when changing its direction, and Fig. 5 when passing between objects too large to be moved.

FIGS. 6–10.—Degenerating stages of the organism. So-called "budding forms."

FIGS. 11, 12.—Appearance of organism after 24 hours' incubation at 37° C., 25° C., and 10° C.

FIGS. 13–16.—Death of organisms following the action upon them of normal sodium hydroxide and 5 per cent quinine sulfate solution, Fig. 16 representing the remaining cuticle after complete discharge of its contents.

PLATE 2.

FIGS. 1–4.—Conjugation immediately preceding division of the organisms, Fig. 3 representing actual conjugation.

FIGS. 5–9.—Reproduction by amitotic division, following conjugation.

PLATE 3.

FIG. 1.—Balantidium coli in feces showing a large active parasite and a very small, almost inactive one. ×430 diam.

FIG. 2.—An organism in the resting stage preparatory to becoming encysted. ×430 diam.

FIG. 4.—Small superficial ulcer of the large intestine. Some of the remaining glands of Lieberkühn are atrophied, while others are cystic. ×90 diam.

FIGS. 3, 5, 6.—Balantidium coli in the submucous coat of the large intestine. Figs. 3, 5, ×90 diam. Fig. 6, ×150 diam.

PLATE 4.

FIG. 1.—Balantidium coli in lumen of gland of Luberkuhn. ×250 diam.

FIGS. 2, 3.—Parasites in interglandular supporting tissue. ×250 diam.

FIG. 4.—Balantidium coli in a small vein. ×250 diam.

FIG. 5.—Parasite in submucous coat. Note infiltration of lymphoid and plasma cells about the organism.

FIG. 6.—The parasite in a lymph space. ×250 diam.

PLATE I.

PLATE 2.

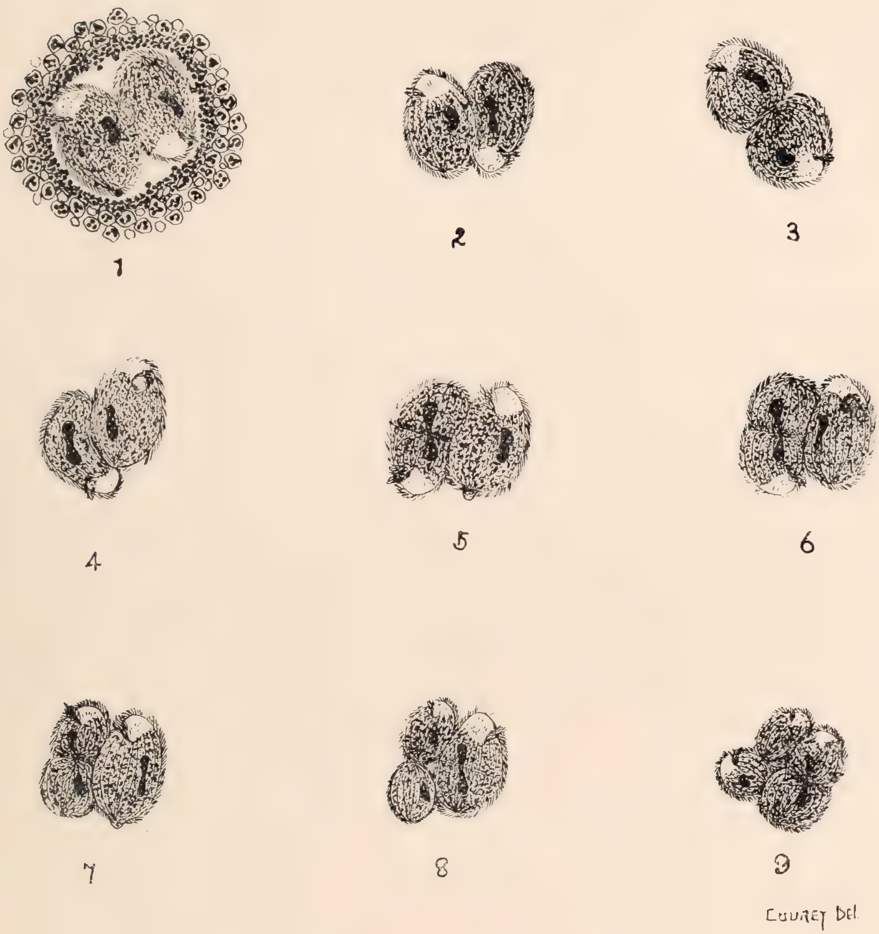
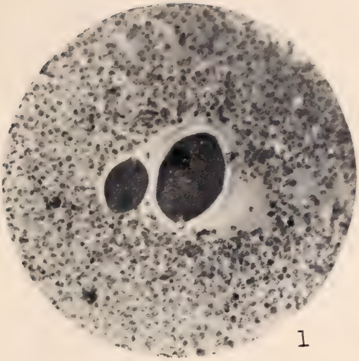


PLATE 3.



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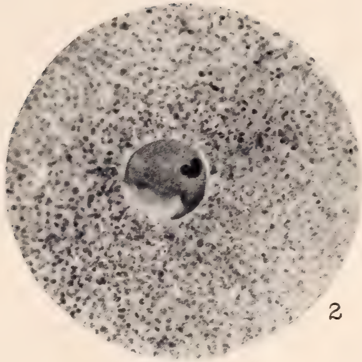

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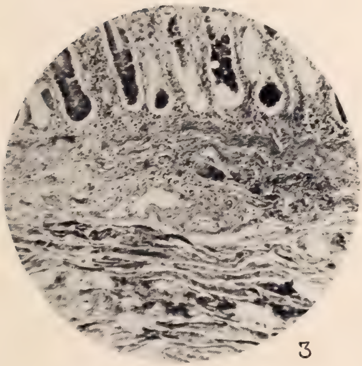

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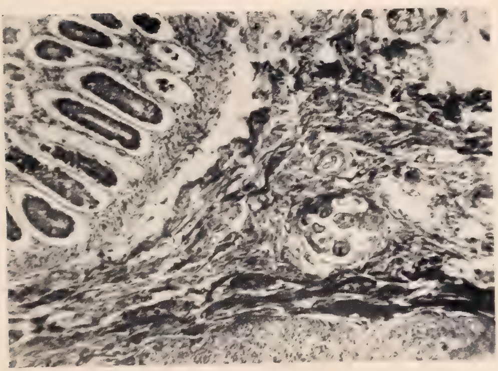

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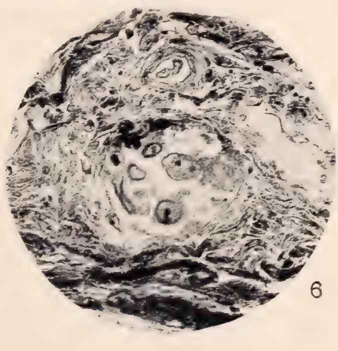

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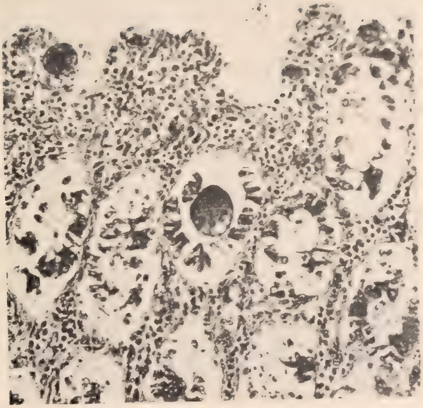
PLATE 4.



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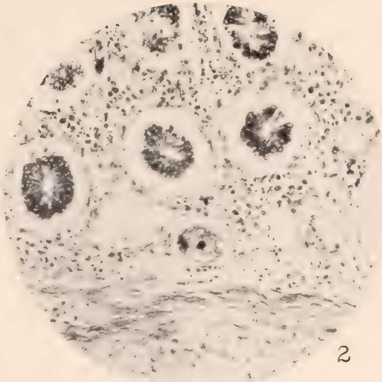

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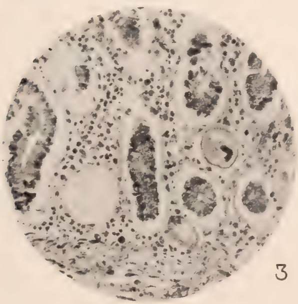

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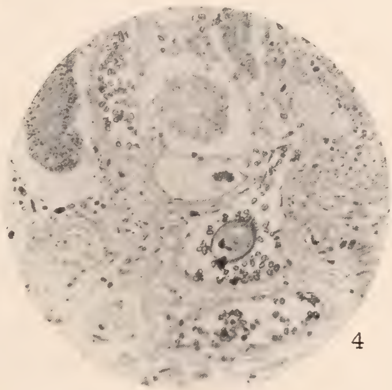

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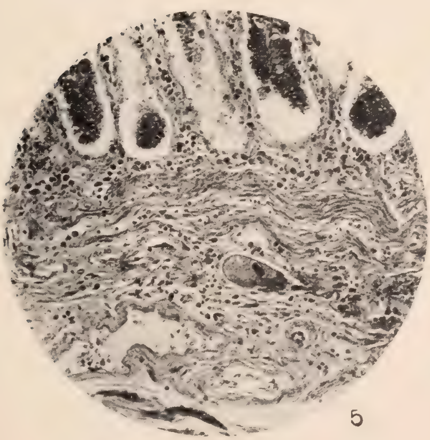

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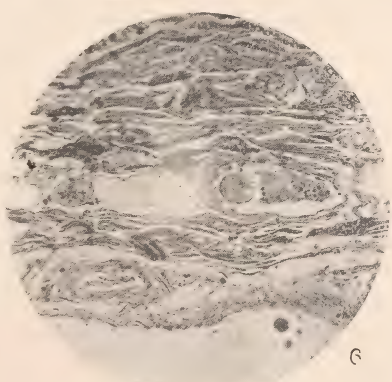

6

THE INFLUENCE OF EXTRACTS OF ANCHYLOSTOMA CANINUM ON THE COAGULATION OF THE BLOOD AND ON HEMOLYSIS.*

LEO LOEB AND MOYER S. FLEISHER.

(From the Laboratory of Experimental Pathology of the University of Pennsylvania.)

IN former experiments, one of us (Loeb, with A. J. Smith) had found that in the anterior part of Anchylostoma caninum a substance is present that has a distinctly inhibiting action upon the coagulation of the blood.[1] At that time we did not carry out experiments concerning the hemolytic action of such extracts, but we noticed that in the mixtures used for the determination of the coagulation time no distinct hemolytic influence of the extract could be observed.

In the posterior part of anchylostoma little of the substance inhibiting the coagulation was present in one experiment, while in the other experiments it was absent. Boiling the extract during a period of 15 minutes caused a marked weakening of its activity, without, however, destroying it entirely.

At that time Loeb had assumed that the substance present in anchylostomum would in all probability be found to be similar to hirudin, the substance of the leech which inhibits coagulation, in fact the analogy which at that time appeared to exist between the behavior of the leech and of anchylostomum induced one of us to undertake these experiments.

In order to test this hypothesis we carried out new experiments. For some time past we searched for specimens of Anchylostoma caninum in all the dogs upon which we performed an autopsy. The anchylostoma found were carefully washed and afterward dried with filter-paper. The worms thus freed from adhering intestinal contents were ground up in a porcelain mortar and the ground up material was dried at a temperature varying between approximately 42° and 50° C. The dried material was scraped off and collected in a bottle for further use. In this way approximately 170 mgm. dried substance of Anchylostoma caninum were obtained, which enabled us to carry out a limited number of experiments.

* Received for publication May 31, 1910.

[1] *Centralbl. f. Bakt.*, 1904, Abt. 1, Orig., 37, p. 93; *ibid.*, 1906, Abt. 1, Orig., 40, p. 738.

THE INFLUENCE OF ANCHYLOSTOMA EXTRACT ON COAGULATION OF THE BLOOD.

Before giving our results, it might be advisable to state briefly the reactions that are characteristic of hirudin. If a moderate quantity of hirudin is added to blood *in vitro*, the plasma obtained after centrifugation coagulates readily under the influence of the tissue-coagulins as they exist in the extracts of the liver and kidney and it also coagulates after the addition of a suspension of mammalian erythrocytes that have previously been hemolyzed. Usually tissue extracts accelerate the coagulation to a high degree if a certain optimal quantity is added; a still larger quantity of tissue extract acts less favorably.

Blood serum has relatively a very slight coagulating effect upon hirudinplasma; but the larger the quantity of blood serum added, the more marked is the accelerating effect upon the coagulation of hirudinplasma. A combination of blood serum and tissue extract acts in a similar manner as tissue extract alone or slightly better, if it is used immediately after the substances have been mixt. If, on the other hand, the combination is used approximately after 10 minutes' standing, the serum exerts a markedly inhibiting action upon the coagulating effect of the tissue extract.

A combination of a suspension of hemolyzed erythrocytes and of blood-serum coagulates more strongly than either alone, especially, if the mixture has been standing for 15 minutes before it is used.

If we now study the action of these various substances upon blood plasma to which a certain amount of anchylostoma extract had been added, we find that the "anchylostomaplasma" obtained by centrifugation of the blood differs in its reactions markedly from the hirudinplasma.

Extract of kidney which exerts a very active coagulating effect upon hirudinplasma is without such influence if added to anchylostomaplasma and it may even inhibit the action of other coagulating agencies. Blood serum, on the other hand, which accelerates the coagulation of the hirudinplasma only to a very slight extent has a very marked coagulating influence upon anchylostomaplasma.

In a combination of serum and kidney extract the kidney extract seems even to possess an inhibiting influence upon the coagulating

power of the serum if both are added to anchylostomaplasma. Hemolyzed dog-erythrocytes, however, exert a pronounced coagulating power upon hirudin as well as upon anchylostomaplasma and a combination of serum and erythrocytes which had been kept at room temperature for 10 minutes before being added to the anchylostomaplasma was extremely active.

The addition of various quantities of $CaCl_2$ to the anchylostomaplasma either alone or in combination with kidney extract did not cause coagulation. (0.1 and 0.2 c.c. of a 1.22 per cent $CaCl_2$ solution were added to 1 c.c. of anchylostomaplasma or to a mixture of 2 c.c. containing 1 c.c. anchylostomaplasma and various quantities of kidney extract and 0.85 per cent NaCl solution.)

Addition of an equal amount of distilled water to the anchylostomaplasma caused a very slow coagulation. Three hours after the addition of the water no trace of coagulation was noticeable. Six hours after the addition of the distilled water the coagulation was not quite complete. It must, however, be stated that the distilled water had been added on the day following the preparation of the plasma.

In regard to the quantities of anchylostoma-powder needed, the following observations were made: In one experiment 85 mgm. were suspended in 6 c.c. of a 0.85 per cent NaCl solution. In this mixture, which reacted approximately neutral to litmus-paper 14 c.c. dog-blood were received with the aid of a canula introduced in a femoral artery. The plasma obtained from this mixture remained entirely liquid for 24 hours; but it would probably have remained still longer in a liquid state, if some substances causing coagulation had not been added at various periods. If we consider that the powder represents the substance of the whole body of the anchylostoma, while the active substance is produced only in the anterior part of the body (probably in one of the glands), furthermore, that the largest part of the powder is entirely insoluble in water or in blood plasma and that the active substance can have represented only a very small fraction of the powder, we are justified in the conclusion that the substance exerts a strong anticoagulating influence.

In another experiment 13 mgm. of anchylostoma-powder were suspended in 5 c.c. NaCl solution. In this case the control-blood collected in an equal amount of 0.85 per cent NaCl solution coagu-

lated in 2–2½ minutes, while 2 c.c. blood mixt with 2 c.c. of the anchylostoma suspension coagulated in 39 minutes. In this case the quantity of anchylostoma used was much smaller and the erythrocytes which remained in contact with the plasma exerted a coagulating influence upon the fibrinogen.

A part of the protocol of Experiment 2 may be given as an example:

D. A. P. = Dog-plasma + anchylostoma extract
D. X. = Dog-kidney extract
D. S. = Dog-blood serum
D. C. P. = Dog-erythrocytes

1 c.c. D. A. P. +	1 c.c.	D. X.	not yet coagulated	22 hours
1 c.c. D. A. P. +	0.5 "	D. X.	" " "	22 "
1 c.c. D. A. P. +	0.3 "	D. X.	" " "	22 "
1 c.c. D. A. P. control			" " "	22 "
1 c.c. D. A. P. +	2 c.c.	D. S.	coagulated	35 minutes
1 c.c. D. A. P. +	0.7 "	D. S.	"	1 hour 20 minutes
1 c.c. D. A. P. +	0.3 "	D. S.	"	4 " 20 "
1 c.c. D. A. P. +	0.3 c.c.	D. C. P.	"	1 " 38 "

1 c.c. D. A. P. + [0.7 c.c. D. S. + 0.3 c.c. D. X. being mixt and added immediately to D. A. P.] coagulated 2 hours 58 minutes.

1 c.c. D. A. P. + [0.7 c.c. D. S. + 0.3 c.c. D. C. P. 10 minutes after mixing added to D. A. P.] coagulated 1 minute 50 seconds.

We may summarize as follows the principal facts which we found in our study of the action of anchylostoma upon the coagulation of the blood:

1. In Anchylostoma caninum a substance is produced inhibiting the coagulation of the blood *in vitro.*

2. This substance is formed in the anterior part of the body of anchylostoma, presumably in one of its glands.

3. This substance is not destroyed, but markedly weakened by boiling during a period of 15 minutes.

4. It can be preserved for a long time by drying the anchylostomas and then converting them into a powder.

5. After the addition of a sufficient quantity of anchylostoma extract the blood-plasma does not, or only with great difficulty, coagulate after the addition of tissue extract; it coagulates relatively readily after the addition of blood-serum and of hemolyzed erythrocytes.

6. A combination of erythrocytes and serum proved to be extremely active.

7. Addition of $CaCl_2$ to the plasma alone or to a combination of plasma and tissue extract does not cause coagulation.

8. On the second day dilution with distilled water led to a very slow coagulation of the plasma, but in this case the possibility could not be excluded that at that time the plasma was near the point of spontaneous coagulation; lack of material prevented us from carrying out desirable control tests in this case.

From these facts we can draw the conclusion that the inhibiting substance in anchylostoma is not analogous to hirudin. We have at present no indication that the inhibiting substance acts by inactivating calcium; in consideration of the small quantities of the substance needed for obtaining an anticoagulating effect, we can exclude the hypothesis that the action is due to the presence of sufficient quantities of the common inorganic salts which, in themselves, exert a certain inhibiting influence.

Altho at the present time we cannot make any positive statement concerning the nature of this substance, we wish to draw attention to a certain similarity which seems to exist between the action of cobra-venom and anchylostoma extract upon the coagulation of the blood. According to Morawitz[1] after the addition of cobra-venom, the plasma also coagulates relatively more easily under the influence of serum than of tissue extract.

Weinberg[2] found that Sclerostomum equinum in many respects behaves in a manner similar to anchylostoma and this investigator found also that extract of Sclerostomum equinum inhibits markedly the coagulation of the blood. It would be of interest to examine the character of the anticoagulating substance of sclerostomum and to compare it with that of anchylostoma. The former may also be found not to resemble hirudin.

DOES ANCHYLOSTOMA EXTRACT CAUSE HEMOLYSIS?

In our previous experiments in which we made use of the extracts of fresh anchylostoma we found no noticeable hemolysis in our blood-anchylostoma extract mixture. At that time, however, we had been mainly concerned with the effect of the extracts upon the coagulation

[1] *Deutsches Archiv f. klin. Med.*, 1904, 80, p. 340.

[2] *Ann. de l'Inst. Pasteur*, 1907, 21, pp. 417, 531.

of the blood and not upon hemolysis; and we had not carried out experiments in which we used washed erythrocytes.

In this series we carried out some additional experiments in which we tested the extracts of anchylostoma powder (which we had obtained in the manner indicated above) upon erythrocytes of the dog, freed from serum through washing with 0.85 per cent NaCl and by centrifugalizing and by repeating this process four times.

The results of these experiments can be briefly stated. We added 5 mgm. or less of the anchylostoma powder to 1 c.c. of a 5 per cent suspension of washed dog-erythrocytes. This quantity of powder did not exert the slightest hemolytic effect upon the erythrocytes of the dog. After this fact had been established we tested combinations of lecithin and anchylostoma extract. Lecithin when used in doses in which it alone did not exert a hemolytic effect did not activate anchylostoma extracts.

The anchylostoma extract contained 5 mgm. of anchylostoma powder in each c.c. of 0.85 per cent NaCl solution, and the lecithin solution was prepared by diluting a 1 per cent solution of Kahlbaum's lecithin in methylalcohol 100 times with a 0.85 per cent NaCl solution. As much as 1 c.c. of the anchylostoma extract was used in combination with 2.5 c.c. and with smaller quantities of the lecithin suspension, without producing any more hemolysis than in the control, in which lecithin alone had been used or in which merely 0.85 per cent NaCl solution had been added to the erythrocytes. The same quantity of anchylostoma powder exerts an extremely strong inhibiting influence on the coagulation of 1 c.c. blood-plasma.

In interpreting these results we have to admit the possibility that the drying of the anchylostoma substance at a temperature varying between 42° and 50° C. may have destroyed a hemolysin which might possibly have been present. This is, however, a not very probable assumption, if we consider that the degree of heat increase was slight and that the amount of water in the anchylostoma powder was reduced to a minimum within a very short time.

Weinberg found in sclerostomum a hemolytic substance, soluble in NaCl solution and resistant to heating to 115–20° C. during a period of 15–20 minutes. It is not very probable that in our case the drying process which took place between 42° and 50° should have destroyed

the hemolysin. Whipple[1] believes that only a very weak hemolysin is present in anchylostoma extracts; he did not observe a hemolytic action if less than 20 anchylostoma were extracted in 1 c.c. of 0.852 per cent NaCl. Our experiments and those of Whipple prove that the hemolytic action of anchylostoma must be very weak indeed, if at all present. Preti found[2] in anchylostoma a hemolytic substance that was not soluble in a physiological NaCl solution, but was soluble in ether and alcohol; it was heat-resisting. Preti evidently did not observe a hemolytic effect of the extract made by means of a 0.85 per cent NaCl solution. The etiological significance of the hemolytic action of anchylostoma appears to be very doubtful, especially if we consider that certain normal organs as well as the contents of the small intestines contain hemolytic substances without causing anemia. If hemolysis plays any part in the anemia of uncinariasis, it might as well be caused by an absorption of certain parts of the contents of the small intestines through the injured intestinal wall as by the very weak hemolytic action of anchylostoma.

However that may be, we can be certain that the anchylostoma contains a substance inhibiting the coagulation of the blood and that this substance favors probably the continued bleeding following an injury of the intestinal wall; and that the presence of such a substance is the more important because the intestinal mucosa would exert a very marked coagulating action upon the blood and no after-bleeding would take place, without the existence of a special anti-coagulating substance.

CONCLUSIONS.

1. In the anterior part of anchylostoma a substance is present that inhibits the coagulation of the blood; it can be preserved for a long time in a dried condition. It is not analogous to hirudin, but it seems to show some similarity to the substance inhibiting the coagulation of the blood which is present in cobra-venom. It will, however, be necessary to make additional comparative tests before such a relationship can be considered proven.

2. The powder of anchylostoma which we used did not contain a direct hemolytic substance, nor a substance which can be activated through a combination with lecithin.

[1] *Jour. Exper. Med.*, 1909, 11, p. 331. [2] *Münch. med. Wchnschr.*, 1908, 55, p. 436.

THE DETERMINATION OF THE NUMBER OF BODY CELLS IN MILK BY A DIRECT METHOD.*

S. C. Prescott and R. S. Breed.

For some time sanitarians have felt that it was important to be able to determine the number of body cells[1] in milk. Large numbers have been held to be undesirable inasmuch as such conditions seem to be associated with abnormal conditions of the udder.

At the present time, the method in most general use in Board of Health and similar laboratories for determining the number of these body cells present in a given sample of milk is the so-called "smeared sediment" method first devised by Stokes and Wegefarth[2] of Baltimore. Later this method was modified by Stewart[3] of Philadelphia and Slack[4] of Boston. These modifications have improved the accuracy of the method but the results obtained with it have not been entirely satisfactory.

In 1905, a "volumetric" method for determining the number of body cells was suggested by Doane and Buckley.[5] This method uses the well-known Thoma-Zeiss hemacytometer for examining the sediment secured by the use of the centrifuge. Practically the same method was devised independently by Savage.[6] The results obtained by this method show larger numbers of body cells present than do those obtained by the "smeared sediment" method. Nearly all of the research work along this line is being done by the "volumetric" method because there seems to be general agreement that it is more accurate than the "smeared sediment" method. Russell and Hoffman[7] find that they get better

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[1] On account of the recent work claiming that the cellular elements present in milk are not leukocytes but detached epithelial cells, the noncommittal term "body cells" will be used throughout this paper instead of the more common expressions "leukocytes" or "pus" cells.

[2] *Med. News*, 1897, 71, p. 45.

[3] *Amer. Med.*, 1905, 9, p. 486.

[4] *Jour. Infect. Dis.*, 1906, Supplement No. 2, p. 214.

[5] *Md. Agri. Exper. Sta. Bull.*, 1905, 102, p. 205.

[6] *Jour. Hyg.*, 1906, 6, p. 123.

[7] *Wis. Agri. Exper. Sta. 24th Ann. Rep.*, 1907, p. 231.

results by heating their samples before centrifuging, and their recent work has been done in this way.

A method whereby it is possible to determine the amount of sediment present in milk and indirectly the number of body cells has been proposed by Trommsdorff.[1] This uses the well-known principle of the hematocrit. It has not found general favor in this country.

THE NEW METHOD.

During the summer of 1909, an entirely new method for determining the number of body cells present in milk was devised in the Boston Bio-Chemical Laboratory. By this method, the number of cells present is determined by a direct examination of the milk without recourse to the centrifuge, thereby simplifying the operation and also avoiding the very serious error which must be present in any method based on the use of the centrifuge. Without going into the details of the steps which led up to the devising of this method, it may be described as follows:

The sample is well shaken to distribute the cream equally through the milk. A measured drop (0.01 c.c.) is then withdrawn by means of a specially constructed capillary pipette with a rubber bulb.[2] This drop is then spread evenly over an area of 1 sq. cm. on an ordinary glass slide. The milk is then dried with gentle heat, the fat dissolved out with xylol or other fat solvent, the smear fixt to the slide by immersion in alcohol for a few minutes, dried, overstained with methylene blue, and decolorized with alcohol. The slide is then ready for examination.

When well done the smear presents an even appearance and the cells show up clearly on a bluish field. This background is the dried casein and other milk solids, and shows holes where the fat drops have been removed. Some difficulty was experienced at first in getting the smears to stick to the slide, but since the adoption of the method of treating with alcohol before staining there has been no trouble of this sort. The cells are evenly distributed even at the edges where there is frequently a somewhat

[1] *Berlin Tierarztl. Wchnschr.*, 1906, p. 81.

[2] May be obtained of Bausch and Lomb Optical Co., Rochester, N.Y., at a cost of fifty cents each.

thicker border of milk solids. Rapid drying is necessary in order to prevent the segregation of the fat drops, a thing which tends to make the distribution of the cells uneven.

Where large numbers of examinations are to be made, it will be found convenient to use the 2×8 inch slides now in use in many laboratories using the "smeared sediment" method. A convenient way to determine the 1 sq. cm. area is to place the slide to be used over one of the ruled plates used in counting bacterial colonies. It will be found somewhat difficult to get even smears in all cases, so that smears should always be made in duplicate.

The determination of the number of cells present is made with the microscope, using the oil-immersion lens. If the diameter of the field is so arranged that it equals 0.16 mm., then each field covers approximately 0.005 of a square centimeter. This adjustment may be made by the use of the draw tube of the microscope. On this basis each cell seen in a field taken at random represents 500,000 cells per c.c. If 100 fields are counted and the total number of cells seen be obtained, then each cell represents 5,000 cells per c.c. This reduces the probable error to a smaller amount than would be necessary for routine work. If the smears are well prepared, the cells may be readily seen and counted with lower powers of the microscope, thus making the counting less laborious.

A series of 31 tests done in duplicate where 100 fields of the oil-immersion lens were counted on each smear shows a variation of 14.5 per cent. In this series of tests, there are two cases with an error greater than 30 per cent (i.e., 42.9 per cent and 64.3 per cent), but both of these were cases where the cellular content was less than 250,000 per c.c. In such cases, the number of cells present in the smears is so few that an error of 50 per cent or more is not surprising, and is of little practical consequence.

The error seems to be due to inaccuracy of count rather than in the preparation of the smear. Even on the same smear a difference of 15 per cent may occur in two different counts. The error is materially lessened if the test is done in duplicate, 100 fields counted on each smear, and the results averaged. All of the results given in this paper were obtained in this way.

DISTRIBUTION OF THE BODY CELLS IN CENTRIFUGED SAMPLES.

A careful examination of centrifuged samples of milk reveals the fact that the use of this instrument introduces so large and so variable an error that it practically vitiates the results obtained by both the "smeared sediment" and "volumetric" methods. It has usually been assumed, without sufficient ground for the assumption, that the centrifuge preciptates all but a small fraction of the cells and that whatever loss there is represents a fairly constant proportion of the whole. Savage made some investigation of the matter and reports that recentrifuging shows that approximately 12 per cent are lost and also that this fraction appears to be practically constant. He does not seem to have considered the possibility that he lost any large number of the cells by discarding the cream. Russell and Hoffman have followed up the question farther. They find that the number of cells precipitated in the sediment after centrifuging is increased by heating the milk to 60° C. They also state that they find more cells in the cream than in the skim milk where the cream was raised by gravity. Apparently this determination of the number of cells present in the cream was made by centrifuging the cream and determining the number of cells present by the "volumetric" method. If this was the case, the number of cells present must have been much larger than they observed, for, as we shall show, many would have been lost in the discarded cream.

An examination of centrifuged sample shows the following conditions:

Sample 1.—The milk used was taken from a quart bottle of market milk obtained from a first-class dealer. This dealer is one who has had the milk from all of his herds examined frequently for high cell content by the "smeared sediment" method. Where high numbers have been detected, the cows have been carefully examined by a veterinarian and the offending cow or cows removed from the herd.

a) The number of cells present was determined by the "direct" method by four tests as 1,440,000, 1,625,000, 1,240,000, and 1,260,000 per c.c. Average = 1,390,000 per c.c. or 2,780,000 per 2 c.c.

b) Seven 2 c.c. samples were centrifuged in the 2 c.c. rubber-stoppered tubes ordinarily used in the "smeared sediment" method (see Stewart) at 3,600 revolutions per minute for 10 minutes.

Numbers one to three of these tubes were used to make smears by the "smeared sediment" method. The smears were spread over an area of 4 sq. cm. and the total

number of cells present in each smear was computed. Fifty to one hundred fields of the oil-immersion lens were counted in each case. No. 1 = 375,000, No. 2 = 165,000, No. 3 = 620,000. Average = 385,000. Some cells must have been lost by adhesion to the stopper, and so it is probable that the average number present in the sediment was 400,000, or approximately one-seventh of the actual number present.

A more thorough examination of the remaining tubes was made to determine the exact position of the remaining six-sevenths of the cells as accurately as possible. Previous experimentation had shown that a large proportion of the cells would be found in the cream, that a number would be present in the milk just under the cream and in the milk just above the sediment, and that the middle region would be comparatively free from the cells. The 2 c.c. tubes were therefore divided into regions as indicated in Fig. 1. In these A = the cream, B = the milk just below the cream (A + B = 1/2 c.c.), C = the middle region (1 c.c.), D = the lower 1/2 c.c., E = the sediment.

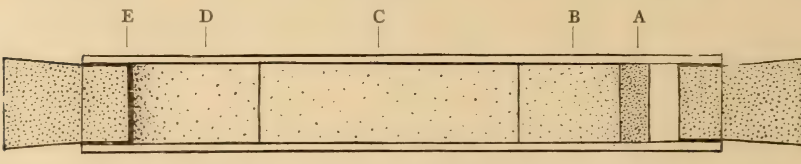

Fig. 1.—A drawing of a double-stoppered glass tube containing 2 c.c. of milk as it shows after being centrifuged. The part of the tube containing the milk is divided by vertical lines into Regions A, B, C, D, and E. A = the cream, B = the milk just below the cream (A + B = ½ c.c.), C = the middle c.c., D = the lower ½ c.c., and E = the sediment.

In Experiment 1, A contained 1,950,000 cells, B = 80,000, C = 50,000, D = 300,000, E = 400,000. Total = 2,780,000.

In Experiment 2, A = 1,330,000, B = 100,000, C = 55,000, D = 500,000, E = 625,000. Total = 2,610,000.

The number of cells present in the cream (A) could not be determined directly. However, an examination of the cream removed from tubes Nos. 1 and 2 was made by dissolving the cream in each case in 2 c.c. of ether in the rubber-stoppered tubes. The tubes were then recentrifuged. As a result of this treatment the cream appeared in the form of a nearly transparent jelly-like substance in the bottom of the tube. An examination of this jelly was made by drying some of it on a slide and then staining in methylene blue. It was impossible to make any accurate estimate of the number of cells present, but it was apparent that 1,000,000 or more were present.

The number of cells present in regions B and C was determined by the "direct" method in Tubes 4 and 5. Region B = 80,000 (average of 4 determinations). Region C = 50,000 (average of 12 determinations).

The number of cells present in Regions D and E in Tubes 4–7 was determined by a method which gives a good idea of the number actually present in the two regions combined, as some 20 previous tests on similar samples had shown. After all of the milk and cream had been removed from Regions A, B, and C, each tube was again filled with water and recentrifuged. This was done to reduce the amount of casein and other milk solids present in Region D by dilution and yet without appreciable loss of cells. All but 1/2 c.c. of the material in each tube was then discarded, the sediment thoroughly mixt with the remaining 1/2 c.c., and the whole spread evenly over an area of 4 sq. cm. on a glass slide and dried with gentle heat. After staining the smear with

methylene blue, the total number of cells present was determined by the use of the oil-immersion lens in each of the four smears as follows: No. 4 = 620,000, No. 5 = 735,-000, No. 6 = 715,000, No. 7 = 500,000. Average = 640,000. It has been arbitrarily assumed that enough cells were lost in the necessary manipulations to make the actual number 700,000. If the determination of the number of cells for the sediment (E) be taken as 400,000 as given above, then the number of cells for D is 300,000.

The numbers and distribution of the cells in the centrifuged samples are shown graphically in Fig. 1 already referred to. The number given for the cream (A) was obtained by subtraction from the total.

Sample 2.—A second sample of market milk obtained from an equally high-grade dealer was examined in a similar fashion.

a) Number of cells as determined by the "direct" method, four determinations: 1,350,000, 1,365,000, 1,280,000, 1,230,000. Average, 1,305,000 per c.c. or 2,610,000 per 2 c.c.

b) Two determinations were made by the "smeared sediment" method. 615,000 cells were found in the sediment of one 2 c.c. tube and 620,000 cells in the sediment of the other tube. Average, 617,000, or if loss due to manipulation be added, 625,000.

The average of four determinations made by the "direct" method showed the number of cells for Region B to be approximately 100,000.

The average of nine determinations by the same method showed 55,000 cells in Region C.

No direct determination of the number of cells present in Region D was made, but the number was estimated at 500,000. This estimate is unsatisfactory but is based on the result of some twenty or more similar examinations and may be regarded as approximately correct.

The number of cells present in the cream (A) was obtained by subtraction.

There are many imperfections in these determinations, and others are to be made, but the results are very suggestive. Moreover, the fact that this distribution of the cells in the milk after centrifuging corresponds with the known distribution of bacteria in similar samples as well as other data, indicates that these results may be relied upon. If so, it is clearly evident that no method of estimating the number of cells present, which is based upon the use of the centrifuge, can be relied upon to give even an approximate idea of the total number of cells present. The fact that the percentage of cream present in different samples of milk varies greatly, raises the question as to whether milks rich in butter fat would not show a relatively lower cell content than milks which are low in butter fat, if the "smeared sediment" or "volumetric" tests be used.

It will be noted that in one of these samples only one-seventh of the total number of cells present appeared in the sediment

obtained by centrifuging, while in the other case nearly one-fourth appeared in the sediment. To test the question whether the ratio between the number of cells actually present and those obtained by centrifuging remained approximately constant, a series of 22 comparative tests were made using the "smeared sediment" and "direct" methods. The samples used were obtained from eight half-pint cans as they were delivered to the milk station from the farms supplying one of the high-grade dealers referred to above. Each sample is the mixt milk of several cows.

Smeared Sediment Method No. of Cells per c.c.	Direct Method No. of Cells per c.c.	Ratio
415,000	2,000,000	1: 4.8
40,000	640,000	1:16.0
75,000	430,000	1: 5.7
65,000	290,000	1: 4.5
95,000	330,000	1: 3.5
150,000	860,000	1: 5.8
245,000	850,000	1: 3.4
15,000	60,000	1: 4.0
115,000	310,000	1: 2.7
65,000	190,000	1: 3.0
1,650,000	10,690,000	1: 6.5
185,000	3,160,000	1:17.1
485,000	1,140,000	1: 2.4
230,000	610,000	1: 2.6
40,000	170,000	1: 4.3
90,000	3,710,000	1:41.2
340,000	2,830,000	1: 8.3
250,000	1,880,000	1: 7.5
1,060,000	2,310,000	1: 2.2
280,000	2,880,000	1:10.3
75,000	330,000	1: 4.4
775,000	1,540,000	1: 2
6,740,000	37,210,000	1: 5.2
Average = 305,000	1,690,000	

It will be seen from these tests that sometimes as many as one-half of the cells are present in the sediment while at other times not over one-fortieth of the total number are present. Under these circumstances, it is little wonder that the Committee on Standard Methods of Bacterial Milk Analysis appointed by the American Public Health Association recommends that little direct weight be given to the leukocyte tests now in use.

The fact that the number of cells present in the sediment can be materially increased by heating the milk to 60° C. before cen-

trifuging as found by Russell and Hoffman is explained by these results. The heating changes the physical nature of the milk in such a way that more of the cells are precipitated and fewer go to the cream.

The fact that the results obtained by the "volumetric" method are higher than those obtained by the "smeared sediment" method is apparently due to the use of the milk just above the sediment as well as the sediment itself.

NORMAL NUMBER OF BODY CELLS PRESENT IN MILK.

A large series of determinations of the numbers of cells present in milk using this new method or some other direct method of examination must be made before it is possible to state the normal maximum. In addition to the 22 tests already given, 24 others were made of similar samples. In these as well as in the former case, the numbers given represent the average of two determinations in which 100 fields of the microscope were counted for each smear. No two of these samples came from the same farm, so that the combined results of the two tables give the conditions that obtain for 46 New England farms supplying milk to Boston. The samples were obtained during the month of August when the cattle were living on rations composed largely of grass supplemented by some grain or corn fodder.

NUMBER OF CELLS PER C.C. IN 24 SAMPLES.

90,000	3,620,000	210,000
1,660,000	1,430,000	1,100,000
230,000	2,010,000	660,000
80,000	1,580,000	2,330,000
810,000	1,580,000	3,610,000
690,000	820,000	1,500,000
540,000	750,000	170,000
900,000	1,410,000	3,380,000

Average of the 46 tests = 1,485,000 per c.c.

A series of examinations was made of quart bottles of market milk after they had been carried about the city of Boston on the regular morning delivery. It should be stated that seven of the eight samples were obtained from the same milk station and on the same morning. All of these samples were reported O.K. (i.e., showed less than 10 cells per field by the "smeared sediment"

method) in the routine examination made in the laboratory. Samples obtained August 17 and 23, 1909:

1,310,000	2,290,000	2,170,000
3,414,000	1,390,000	2,510,000
5,210,000	4,480,000	

Average of eight tests, 2,850,000 per c.c.

CONCLUSION AND SUMMARY.

1. The centrifuge precipitates between 2.5 per cent and 50 per cent of the total number of body cells present in milk. A greater percentage of these cells usually rises with the cream. The skim milk is practically free from them except just under the cream and just above the sediment.

2. The percentage of cells precipitated is so variable that no reliable estimate of the total number of cells present can be made from a determination of the number found in the sediment.

3. Therefore, no method based on the use of the centrifuge can be satisfactory.

4. A method of enumerating the number of cells in milk by an examination of smears of dried milk, made by using a definite quantity of milk and spreading it over a definite area, has been devised. The counting of the cells is done by means of the microscope after staining the smear.

5. A series of tests made by this method shows that the average number of these cells present in milk is approximately 1,500,000 per c.c. This number is far greater than has previously been supposed to be present, and the present standard (500,000) will need to be greatly increased if any numerical standard is retained. Very few samples contain less than 100,000 cells per c.c. One test of milk having a normal appearance and sold as market milk showed 10,690,000 cells per c.c.

FORMALDEHYDE DISINFECTION WITH SPECIAL REFERENCE TO THE COMPARATIVE VALUE OF SOME OF THE PROPRIETARY PRODUCTS.*

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At a recent meeting of the American Public Health Association, B. R. Rickards[1] reported the result of an investigation of the methods of disinfection employed in the larger cities of the United States. He had sent a circular letter to every city of over 100,000 and received replies from 29 cities. The methods employed in these cities were as follows:

Formaldehyde generators		9
Generators alone	5	
Combined with other methods	4	
Permanganate-formalin method		12
Permanganate method alone	9	
Combined with other methods	3	
Solidified formaldehyde (proprietary articles sold under various names)		9
Solidified formaldehyde alone	4	
Combined with other methods	5	
Formalin sheet spraying method		4
Sheet spraying alone	2	
Combined with other methods	2	
Formalin-aluminum-sulphate-lime		1
Sulphur		2
Sulphur alone	1	
Combined with other methods	1	

The time of exposure varied from 3 to 24 hours, the majority requiring 6 to 8 hours. All advised sealing the rooms. Seven cities appreciated the value of moisture. One did not deem it advisable. Seventeen cities tested the result of disinfection by exposing cultures in the rooms. Nine bought their formaldehyde on analysis. The amount of formaldehyde used per 1,000 cu. ft. varied from 1 oz. of the solid product to 32 oz. of formalin.

In commenting on these figures, Rickards calls attention to "the large number which use the solid proprietary forms about which they evidently know little except what the agent has told them," and says

* Received for publication April 10, 1910.

[1] *Amer. Jour. Public Hygiene*, 1909, 19, p. 367.

further: "One fact stands out strikingly: each city is proceeding on a go-as-you-please policy, adopting that method which happens to suit the immediate situation best from a standpoint of convenience and expense, regardless usually of the question of efficiency." "The present situation is intolerable viewed from a scientific standpoint. If disinfection is of value in any case, then it should be done in an efficient manner; a manner proved by exhaustive work to be reliable. If disinfection in general is not of value and can be proved to be of little or no value in any given disease, then disinfection in that case should be abandoned and trouble and expense thus saved."

These statements from such eminent authority we believe show fully the necessity for our investigations. If disinfection is "farcical" in the cities investigated by Rickards, then disinfection is farcical in numerous other localities and a revision of the methods recommended by many of the boards of health is distinctly indicated.

In 1906 Daniel Base[1] published the results of a series of experiments performed in the Division of Pharmacology, Hygienic Laboratory, U.S. Public Health and Marine Hospital Service, for the purpose of determining the yield of formaldehyde in various methods of liberating the gas for the disinfection of rooms. These experiments were performed with a room containing 2,000 cu. ft. of space. The room was lined on the sides and ceiling, as well as the floor, with sheet zinc to prevent condensation of the gas upon the surfaces. From these experiments based upon the quantity of gas recovered from the air in the room, the various methods investigated were arranged as follows:

1. Trenner Lee retort	47	per cent
2. Autoclave	41.5	"
3. Permanganate-formalin (1–2)	39.15	"
4. Diluted formaldehyde-permanganate	35.1	"
5. Sheet spraying	30.48	"
6. Formalin-aluminum-sulphate-lime	14	"

The high authority of these investigators has left very little room for argument regarding the comparative value of the methods mentioned, but it will be observed that the experimenters did not give any comparative results with the various proprietary forms of solidified formaldehyde now on the market; and it was essentially for the

[1] *Jour. Amer. Chem. Soc.*, 1906, 28, p. 964.

purpose of comparing these with formalin that our experiments have been undertaken.

Since we did not have at our disposal a zinc lined room and were obliged to employ a room with papered walls and ceiling and wooden floors, the average quantity of gas recovered from the air was necessarily somewhat lower than that obtained by Daniel Base. This was because of the marked condensation of formaldehyde upon the various surfaces. Von Bronn[1] in 1899 made experiments charging an ordinary living room well sealed, with known quantities of formaldehyde. He made three determinations, and the highest percentage of the original formaldehyde charged into the room obtained from the air was 16.94 per cent. The remainder of the formaldehyde was found to have condensed on the exposed surfaces. From these results he concluded: "It can be said that the greatest portion of the liberated formaldehyde is condensed at once on the surface of the walls and on the objects in the room. Accordingly the idea that in disinfection the formaldehyde acts as a gas, needs correction. The more experiments have been made with formaldehyde, the more has it been observed that its maximum germicidal effect can only be obtained in the presence of an abundance of water vapor. Therefore, it appears that by vaporizing formaldehyde, we only accomplish a uniform distribution of the disinfectant in space, but that the real efficiency lies not in the formaldehyde gas but in the solution which condenses everywhere on surfaces." The observations of von Brunn have been repeatedly confirmed by other experimenters, but it has been further observed that the percentage of gas recovered from the air in a given room charged with formaldehyde under the same conditions is fairly constant for different quantities and may be employed in estimating the comparative value of the different methods of charging a room.

The room at our disposal measured 19 ft. $\times$ 12½ ft. $\times$ 12½ ft., having two doors and one window. The walls and ceiling were papered, the floor being of hard wood. The window and one of the doors were carefully sealed with paper. The other door was padded with cloth on all its edges so that when closed it would be as tight as any ordinary room could be made for practical disinfection. The capacity

[1] *Ztschr. f. Hyg.*, 1899, 30, p. 201.

of the room was about 2,965 cu. ft., but in order to give the benefit of all doubt to the various manufacturers and for the purpose of convenience, the room was regarded in all our experiments and calculations as containing 3,000 cu. ft. of space.

TABLE 1.

THE AMOUNT OF FORMALDEHYDE ABSORBED AND THE AMOUNT REMAINING IN THE AIR AT VARYING PERIODS AFTER CHARGING THE ROOM.

Number	Temperature ° C.	Percentage of Humidity	Wind Miles	Formaldehyde Product Used per 1,000 Cu. Ft.	Percentage of Strength of Formaldehyde	Method of Liberation	Time Since Starting	Time Required to Draw Air	Liters of Air Drawn	Absolute Formaldehyde Used per 1,000 Cu. Ft. in Gm.	Absorption per Sq. Ft. of Surface in Mgm.		Absolute Formaldehyde Found per Cu. Ft. of Space in Mgm.	Percentage of Yield
											Moist	Dry		
12	18	85	1	330 c.c.	37.8	$KMnO_4$	10 m.	15 m.	5	125	...	...	32.30	25.90
				Water		330 gm.	1 hr.	1 hr.	20		...	...	16.15	12.96
				270 c.c.			3 hrs.	30 m.	10		...	...	12.32	9.86
							5 "	.....	..		52	23	.....	....
11	19	73	4	330 c.c.	37.8	$KMnO_4$	10 m.	15 m.	5	125	...	...	32.30	25.90
				Water		330 gm.	30 "	.....	..		343	37	.....	.....
				270 c.c.			1 hr.	1 hr.	20		...	...	15.92	12.77
							2 hrs.	.....	..		147	33	.....	.....
							3 hrs.	30 m.	10		56	33	10.62	8.51
							5 "	.....	..		35	30	.....	.....
							24 "	.....	..		19	12	.....	.....
14	21	75	1	300 c.c.	37.8	$KMnO_4$	10 m.	15 m.	5	113	234	21	43.31	38.23
							30 "	.....	..		450	35	.....	.....
						210 gm.	1 hr.	1 hr.	20		...	...	22.50	19.86
							2 hrs.	.....	..		160	33	.....	.....
							3 "	30 m.	10		83	28	17.41	15.30
							5 "	.....	..		45	27	.....	.....
							24 "	.....	..		22	21	.....	.....
9	21	75	6	300 c.c.	37.8	$KMnO_4$	10 m.	15 m.	5	113	197	12	45.01	39.70
						150 gm.	30 "	.....	..		399	22	.....	.....
							1 hr.	1 hr.	20		...	...	22.08	19.50
							2 hrs.	.....	..		163	27	.....	.....
							3 "	30 m.	10		72	24	17.41	15.30
							5 "	.....	..		39	24	.....	.....
							6 "	30 m.	10		...	...	14.01	12.30
23	20	81	4	104	62	Parrafin	45 m.	15 m.	5	64	303	12	30.57	47.50
				gm.		Candle	1 hr.	1 hr.	20		225	24	22.52	35.00
				(Solid)		(DePree)	2 "	.....	..		168	26	.....	.....
							3 "	30 m.	10		75	25	13.60	21.20
							6 "	30 "	10		40	19	11.46	17.80

In comparing the relative value of the different methods of charging the room with formaldehyde, the following points have been considered: (1) the amount of formaldehyde in the air of the room; (2) absorption on moist and dry surfaces; (3) effect on cultures.

Since there is considerable variation in the time required to liberate the formaldehyde by the various methods, an attempt was made to

select an arbitrary time which would insure complete liberation of all the gas before any of the air was drawn for analysis. After a room is charged, the percentage of formaldehyde gas in the air diminishes rather rapidly and the results will be materially lower if the air is drawn some time after charging.

The results show that during the first hour after liberation the amount of gas in the air rapidly diminishes, while at the same time the absorption on dry paper increases. The moist paper shows its highest absorption after 30 minutes, after which the formaldehyde is rapidly lost. This is due to the evaporation of the moisture. After two hours there is a uniform diminution in the amount contained in the air as well as in the dry paper which indicates the rate of leakage.

With one exception, air was drawn in all our experiments, beginning exactly one hour after the charging was begun. This of course favors those preparations which require the longest time to liberate their gas and in comparing results this should be considered.

THE AMOUNT OF FORMALDEHYDE IN THE AIR OF THE ROOM.

After a series of preliminary experiments in which potassium cyanide and iodate solutions were tried for absorbing the gas, it was decided to abandon both and use distilled water. The formaldehyde was absorbed in four 100 c.c. flasks, each containing 50 c.c. of distilled water. The amount of formaldehyde was determined separately in these flasks after the desired quantity of air from the room had been drawn through. Practically all of the formaldehyde was found each time in the first flask and only a small amount in the second. The third and fourth flasks never contained measurable quantities and for practical purposes could, we believe, have been discarded.

The air was drawn from the charged room through a hole in the door which was fitted with a perforated cork stopper through which a glass tube projected about 18 inches into the room. The air was measured by displacement of water in a bottle graduated from the top. All connections and stoppers were rubber and each time before drawing air, clamp No. 1 was closed and clamp No. 2 opened to insure against all leaks. After drawing the air, the flasks were at once disconnected and an accurately measured quantity of $\frac{n}{10}$ iodine added to each one. This was followed by $\frac{n}{1}$ sodium hydroxide, using 1 c.c. $\frac{n}{1}$ NaOH for every 5 c.c. $\frac{n}{10}$ iodine. This was allowed to stand

for about 15 minutes in order to complete the reaction which takes place essentially as follows:

$$I_2 - CH_2O - 2NaOH = CH_2O_2 - 2NaI - H_2O.$$

After 15 minutes, $\frac{N}{1}$ H_2SO_4 in slight excess was run in to liberate the uncombined iodine which was determined by means of $\frac{N}{10}$ sodium thiosulphate using starch indicator. From this titration the number of c.c. of $\frac{N}{10}$ iodine absorbed multiplied by 0.0015 gives the weight of absolute formaldehyde present in the amount of air drawn.

Example: Volume of air drawn $-$20 liters

Amount of $\frac{N}{10}$ iodine absorbed $-$6 c.c.

$$\frac{6 \times 0.0015}{20} = \text{gm. } CH_2O \text{ in 1 liter}$$

One cubic foot is 28.315 liters

$$\therefore \frac{6 \times 0.0015}{20} \times 28.315 = 0.01274 \text{ gm. per cu. ft.}$$

ABSORPTION ON MOIST AND DRY SURFACES.

For determining absorption, Swedish filter papers diam. 9 cm. were used. These were suspended from strings near the sides of the room. The dry papers were taken directly from the package in the laboratory. The moist papers were dipped in distilled water immediately before starting the experiment. These moist papers when removed after five hours were always apparently dry. Nevertheless, the amount of formaldehyde they contained was invariably higher than the amount contained in the dry paper, and the excess of formaldehyde on an average day was probably proportionate to the moisture retained. The absorption was determined after five hours except where otherwise stated.

EFFECT ON CULTURES.

The effect of the various preparations on cultures may best be taken up under three different heads: (*a*) effect on different species; (*b*) time required to kill; (*c*) penetrating power. For the effect on different species, we used staphylococci—obtained from an abscess; streptococci—obtained from throat culture; B. coli—isolated from feces; B. diphtheria—obtained from throat culture; B. typhosus—obtained from Parke, Davis & Co.; B. anthracis—obtained from Parke, Davis & Co. All test cultures were grown in +1 broth 48 hours at 37° C., after which time silk threads about an inch in

length previously sterilized at 160° C. for one hour were dipped in the growth and dried in an incubator at 37° C. for 16 to 18 hours. In the various experiments, "dry cultures" applies to such contaminated threads coming from the incubator and "moist cultures" refers to these same threads moistened with sterile distilled water immediately before charging the room.

The formaldehyde was always liberated in the center of the room and these cultures were suspended by means of an aluminum wire on strings drawn across the room midway between the generators and the wall.

In order to determine the "time required to kill," a string was extended across the room, in position similar to those already described, from a large hole in the door. This string was passed over a pulley at the opposite side and returned, being held in place by a tightly fitting cork. On this string were tied cultures of B. coli which could easily be drawn out one at a time at desired intervals.

Penetrating power was determined by placing layers of common sheeting, after boiling to remove the starch, one above the other. This package was covered on the sides, bottom, and one end with heavy wrapping paper, in which condition it was sterilized in the hot air oven each time before using. Dry cultures were placed between the various layers after which the open end was sealed with paper. This prevented absolutely any formaldehyde from reaching the cultures except as it passed through the various layers of sheeting. After exposure, the threads were placed in sterile +1 broth and incubated for 96 hours at 37° C. except the colon cultures which had been used to determine "time required to kill." These were grown in lactose broth in Smith's fermentation tubes where the organisms could be easily identified and accidental contamination excluded. The following form shows a record of the routine observations made in connection with each experiment.

EXPERIMENT NO. 3.

PERMANGANATE-FORMALIN METHOD. 2 OZ. FORMALIN PER 1,000 CU. FT.

Formalin, 37.8 per cent, 180 c.c.
Permanganate, 90 gm.
Capacity of room, 3,000 cu. ft.
Temperature, 18° C. Humidity, 76. Wind, 8 miles.

Time for reaction, 5 min. Air drawn 1 hr. after starting.
Amount of air drawn, 20 liters. Time, 1 hr.
Amount of absolute formaldehyde used, 68 gm.
Absorption per sq. ft. of surface { Moist, 14.62 mgm. / Dry, 5.62 mgm. }
Amount of formaldehyde found in air { per cu. ft. 2.97 mgm. / entire room, 8.91 gm. }
Percentage of formaldehyde recovered from air, 13.17 per cent.

CULTURAL RESULTS.

48 HR. CULTURES EXPOSED 5 HRS. INCUBATION 96 HRS.

(+ = growth; o = no growth.)

ORGANISMS	MOIST			DRY			CONTROL
	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3	
Staphylococci	o	o	o	+	+	+	+
Streptococci	o	o	o	+	+	+	+
B. coli	o	o	o	+	+	+	+
B. diphtheriae	o	o	o	+	+	+	+
B. typhosus	o	o	o	+	+	+	+
B. anthracis	o	o	o	+	+	+	+

Culture		15 M.	30 M.	1 Hr.	2 Hrs.	3 Hrs.	4 Hrs.	5 Hrs.	
B. coli	Moist	+	o	o	o	o	o	o	growth
		20%	o	o	o	o	o	o	gas
	Dry	+	+	+	+	+	+	+	growth
		25%	30%	20%	15%	30%	40%	35%	gas

B. coli Penetration	Dry	1	2	3	4	5	6	7	8	9	10	11	12	15	Exposure 5 hrs.
		+	+	+	+	+	+	+	+	+	+	+	+	+	

THE PERMANGANATE-FORMALIN METHOD.

When formalin and potassium permanganate are brought together a vigorous reaction takes place, liberating sufficient heat to volatilize a large quantity of formaldehyde gas and water. This reaction begins from 30 to 50 seconds after the ordinary potassium permanganate crystals and formalin are mixt, and rapidly goes on to a strong ebullition which continues until all of the permanganate is reduced or all of the liquid evaporated, which usually requires about five to seven minutes. During the reaction a considerable proportion of the formaldehyde is destroyed by the action of the permanganate, which probably reacts essentially as follows:

$$4KMnO_4 - 3HCOH - H_2O = 4MnO(OH)_2 - 2K_2CO_3 - CO_2.$$

Various experimenters have adopted different proportions of permanganate and formalin in view of obtaining the highest percentage of gas. Evans and Russell[1] recommended $3\frac{3}{4}$ oz. of potassium permanganate to 10 oz. of formalin, but later increased the permanganate to at least $4\frac{3}{4}$ oz. Base and McClintic recommend 5 oz. of potassium permanganate to 10 oz. of formalin. The Bureau of Animal Industry recommended about 8 oz. of potassium permanganate to 10 oz. of formalin. Hill[2] and Roberts recommend seven parts by weight of permanganate to 10 parts by volume of formalin. In choosing from these various formulae, there are two important points for consideration, viz., the amount of absolute formaldehyde available for disinfection and the relative price of the substances employed. A series of experiments was carried out to determine the value of the various proportions with results as follows:

Formula	Amount Destroyed in Reaction	Amount Remaining in Residue after 30 Min.	Amount Available for Disinfection
1. Formalin 10, Permanganate 7	30 per cent	3 per cent	67 per cent
2. Formalin 10, Permanganate 5	22 " "	20 " "	58 " "
3. Formalin 10, Permanganate $3\frac{1}{3}$	15 " "	45 " "	40 " "
4. Formalin 10, Permanganate $2\frac{1}{2}$	11 " "	59 " "	30 " "

The amount of formaldehyde destroyed during the reaction is practically proportionate to the amount of permanganate used, up to certain limits. When the permanganate is increased above the proportion of 7 to 10 the amount of destruction is not proportionate because the heat of the reaction will drive off the formaldehyde before all of the permanganate is reduced. The amount remaining in residue increases very rapidly as the permanganate is decreased and this increase is far from being compensated by the lessened destruction. An increase in the permanganate above the 7 to 10 proportion is not be recommended, for the relative price of permanganate makes further increase uneconomical.

All of the available formaldehyde is not in reality given off in 30 minutes with the lower proportions of permanganate, and in examining residues when using the 1–2 formula after five hours usually

[1] *Fourteenth Report of the State Board of Health of Maine*, 1906, p. 227.

[2] *Amer. Jour. Pub. Hyg.*, 1909, 19, p. 576.

only 5 to 7 per cent of the original formaldehyde is found to remain, and after 24 hours only about 1 per cent. The gas which is given off after 30 minutes, however, is by slow evaporation and has practically no value for disinfection because the rate of leakage from the average room is far greater than such slow evaporation can replace.

Some have recommended various dilutions of the formalin with water for the purpose of increasing the relative humidity. It is a well-known fact that moisture greatly increases the efficiency of formaldehyde as a germicide and when the natural humidity is low and artificial moisture cannot be readily otherwise supplied, this method is to be recommended. In using permanganate-diluted-formalin, two general rules should always be borne in mind: (1) the quantity of permanganate by weight must be at least one-half as much as the entire quantity of liquid by volume; (2) the amount of additional formaldehyde destroyed is proportionate to the increase in the permanganate.

If less permanganate is used than required in Rule I, too large a proportion of the formaldehyde will remain in the residue. If too large a proportion of water be added to the formalin, a proportionate increase of the permanganate is necessary in order to volatilize it and a corresponding destruction of formaldehyde results. Thus if we were to employ a formula like this:

Formalin	10 c.c.
Water	40 "
Permanganate	25 gm.

a fairly dry residue will be obtained, but practically all of the formaldehyde is destroyed.

One of the best formulae for permanganate-diluted-formalin we have seen is that proposed by Hill[1] and Roberts, which is

Formalin	11	parts	by	volume
Permanganate	11	"	"	weight
Water	9	"	"	volume

According to this proportion about 45 per cent of the formaldehyde is destroyed and 5 to 7 per cent left in the residue after 30 minutes, leaving practically 48 to 50 per cent of the formaldehyde available for disinfection in the presence of an abundance of moisture.

[1] *Loc. cit.*

TABLE 2.

EXPERIMENTS WITH THE PERMANGANATE-FORMALIN METHOD.

Experiment Number	Amount Used per 1,000 Cu. Ft.		Temperature ° C.	Humidity Percentage	Amount of Formaldehyde in Mgm. Found per Cu. Ft. of Air Space	Cultural Results: + = Growth, o = No Growth, — = Not Killed in 5 Hrs. — 48 Hr. Cultures, Exposed 5 Hrs. Incubation 96 Hrs. Number of Each Exposed, 3 Moist and 3 Dry															Penetration B. coli Dry Culture
						Staphylococci		Streptococci		B. coli		B. diphtheria		B. typhosus		B. anthracis		Control	B. coli First Culture Killed		
	Formalin, Ounces	Permanganate, Ounces				Moist	Dry	Moist	Dry	Moist	Dry	Moist	Dry	Moist	Dry	Moist	Dry	Dry	Moist	Dry	1. Exposure 5 hrs. 2. " 7 " 3. " 24 "
I.......	½	¼	17	85	85	3+	3+	1+	3+	3+	3+	2+	3+	o	3+	2+	3+	+	—	—	1. None killed
II.......	1	½	20	66	1.48	2+	3+	1+	3+	3+	3+	1+	3+	o	3+	o	3+	+	—	—	1. " "
III.....	2	1	18	76	2.97	o	3+	o	3+	o	3+	o	3+	o	3+	o	3+	+	*30 m.	—	1. " "
IV	4	2	26	70	8.06	o	o	o	o	o	o	o	o	o	o	o	o	+	†15 "	5 hrs.	1. " "
V.......	4	2	22	80	6.58	o	o	o	o	o	1+	o	1+	o	o	o	o	+	†15 "	—	1. " "
VI.. ...	8	4	21	44	12.53	o	o	o	o	o	o	o	o	o	o	o	o	+	†15 "	5 hrs.	1. " "
VII....	8	4	23	82	12.74	o	o	o	o	o	o	o	o	o	o	o	o	+	†15 "	5 "	1. " "
VIII ...	10	5	23	89	15.50	o	o	o	o	o	o	o	o	o	o	o	o	+	†15 "	15 m.	1. 4 layers killed
IX.....	10	5	21	75	22.08	o	o	o	o	o	o	o	o	o	o	o	o	+	†15 "	15 "	2. None killed
X......	16	8	17	66	25.05	o	o	o	o	o	o	o	o	o	o	o	o	+	†15 "	15 "	1. 3 layers killed
XI.....	Water, 9 ounces 11	11	19	73	15.92	o	o	o	o	o	o	o	o	o	o	o	o	+	†15 "	15 "	3. 15 " "
XII	Water, 9 ounces 11	11	18	85	16.15	o	o	o	o	o	o	o	o	o	o	o	o	+	†15 "	15 "	1. None killed
XIII ...	10	7	18	79	16.35	o	o	o	o	o	o	o	o	o	o	o	o	+	†15 "	15 "	1. " "
XIV....	10	7	21	75	22.50	o	o	o	o	o	o	o	o	o	o	o	o	+	†15 "	15 "	3. 2 layers killed

* First culture drawn after starting experiment.

† Second culture drawn after starting experiment.

APPARATUS FOR DISINFECTION.

The Formanganate Disinfector, manufactured by Parke, Davis & Co., Detroit, consists of a 16 fl. oz. bottle of the official Liquor Formaldehyde and a box containing two disks consisting of potassium permanganate, each weighing about four ounces. The outfit is accompanied by directions for use and recommendations for artificially increasing the humidity, as well as other recommendations for the product. The claims made for this preparation are easily substantiated and in the quantity recommended, viz., 16 oz. per 1,000 cu. ft. of space, is undoubtedly efficient. This preparation is practically a duplicate of the formula suggested by McClintic, but the permanganate is in compressed form which causes it to react more slowly than the crystals, and thus eliminates all danger of spattering. A small increase in the formaldehyde obtained after one hour, as compared with the crystals, is pointed out in the manufacturer's circular. This increase also was noticeable in our determinations and is explained, we believe, by the longer time required for the reaction rather than by any increase in the actual amount of gas. The time required to liberate the gas in our experiment was 18 minutes.

DePree's Formaldehyde Fumigator, manufactured by DePree Chemical Co., Holland, Mich., is a solidified formaldehyde having a melting point of about 100° C. and containing a variable proportion of formaldehyde and water, ranging in the samples tested from 62 to 73 per cent formaldehyde. The apparatus consists of a tin box containing the formaldehyde, this being placed over a base containing paraffin having a wick in the center from which the paraffin is burned to evaporate the product. The claim made for this preparation is: "Its efficiency is greater than any of the forms heretofore used, one-half ounce by test being sufficient to thoroughly disinfect 1,000 cu. ft. of space." This preparation is put up in two sizes, No. 0, said to contain one-half ounce for 500 cu. ft. of space, and No. 1, said to contain one ounce for 1,000 cu. ft. of space. Four different lots of these fumigators were examined, the first, consisting of Nos. 1 and 0, was obtained from the general market. The second was obtained from the manufacturers through the Board of Health. The third was taken from a fresh lot at the factory by Secretary Shumway. The fourth was delivered at the laboratory by a representative of the firm for the purpose of these experiments. On examination, these various lots gave the following results:

Lot No.	Size No.	Weight of Product	Formaldehyde Present
1	0	17–18 gm.	66 per cent
1	1	34–35 "	66 " "
2	1	33–35 "	73.2 " "
3	1	35–37 "	63.6 " "
4	1	44–47 "	62.0 " "

All of the samples obtained contained considerably more product than is claimed by the manufacturers, but there seems to be a lack of uniformity both in the amount and formaldehyde strength of the preparations supplied. It is a well-known fact that very small quantities of formaldehyde, under favorable experimental conditions, are capable of destroying certain bacteria, and the claim that one-half ounce in 1,000 cu. ft. of space is sufficient to destroy bacteria is substantiated by our experiments, providing the cultures are moist. On dry cultures, however, this preparation cannot be considered efficient in any of the quantities used in our experiments. Whether further increase in the amount of product would give efficient results on dry cultures was

not determined, because if quantities above 100 gm. per 1,000 cu. ft. are required, the price of this preparation would make its use impractical. Some of the points especially in favor of this preparation are the convenience with which it may be employed and the low melting point, which tends to keep the product evenly distributed in the container until volatilization is complete. Perhaps if artificial moisture can be supplied in a satisfactory manner, this preparation may prove efficient if used in sufficient quantity. The quantity of formaldehyde gas liberated from an average No. 1 DePree candle as obtained in the market is about equivalent to the quantity liberated by three ounces of formalin (40 per cent) when liberated with potassium permanganate, using Hill's 7–10 formula. The time required to evaporate the formaldehyde from a No. 1 candle varied from 35 to 45 minutes.

The International Germ Destroyer, manufactured by International Chemical Co., Chicago, Ill., is a solidified formaldehyde having a melting point of about 170° C. and containing a variable proportion of formaldehyde and water ranging in the samples tested from 57.9 to 69.6 per cent formaldehyde. The apparatus consists of a receptacle of tin containing the product. This container has special provisions for holding added water. The receptacle is placed over a base containing paraffin having a wick in the center from which the paraffin is burned to volatilize the product. This firm claims special efficiency because a certain amount of water is added and volatilized with the formaldehyde. The preparation is put up in five different sizes as follows:

No. 0	said to be sufficient for	1,000	cu. ft.
No. 1	" " " "	2,000	" "
No. 2	" " " "	4,000	" "
No. 3	" " " "	6,000	" "
No. 4	" " " "	8,000	" "

The amount recommended is one and one-half ounce for each 1,000 cu. ft. of space.

Three different samples were examined, all being obtained directly from the manufacturer, with results as follows:

Lot No.	Size No.	Weight of Product	Formaldehyde Present
1	0	39–41 gm.	57.9 per cent
2	0	38–41 "	69.6 " "
Bulk	2 oz.	58–60 "	67.5 " "

A lack of uniformity in the amount of absolute formaldehyde is again manifest. It is evident that the manufacturer's claim or even the actual weight of the product obtained is at best only a crude approximation to the amount of absolute formaldehyde employed.

The preparation, like others of this type, is commendable for the convenience with which it may be used, and special mention might be made of the added water. Artificial moisture does not materially influence the effect on moist or partially moist cultures in experimental work, but when organisms have been thoroughly dried, artificial moisture is unquestionably of considerable value. This preparation does not melt from the heat of the candle but the gas is given off gradually without the product having melted, requiring somewhat more heat than the preparations with lower melting point, and somewhat more charring of the last portion often results. The quantity of formaldehyde gas liberated from one No. 0 International Germ Destroyer as employed in our experiments is about equivalent to the quantity liberated by three ounces of formalin (40 per cent) by the permanganate method, using Hill's 7–10 formula. The

time required to evaporate the formaldehyde from a No. 0 candle varied from 40 to 50 minutes.

Lister's Fumigator, manufactured by Johnson & Johnson, New Brunswick, N.J., is a solidified formaldehyde having a melting point of about 170° C. and containing over 90 per cent of absolute formaldehyde. The apparatus consists of "a candle of solidified formaldehyde inclosed in a fire proof container in such a way that the candle burns from the bottom upward." In using, the product itself is ignited at the bottom and the heat thus generated volatilizes a portion of the formaldehyde which escapes at the top. These fumigators could be obtained in two sizes, each containing about 25 gm. and 50 gm. of product. No definite amount for a given space is specified, but the accompanying circular recommends "one standard size candle for each room" and double that quantity "where bedding, etc., has been gathered into one room." The manufacturers claim that "lighted at the bottom, the candle generates formaldehyde gas in its most active form, which escapes through the top of the container without passing through the flame, and all of the solidified formaldehyde is converted into the active germ destroying gas." In our experiment, three "standard size" candles were used. Moist cultures were killed but none of the dry cultures were affected. About 75 per cent of the gas was destroyed in the process of liberation, which amount we consider abnormal. No further experiments were conducted with this product because the loss was found too great to render the method of value for practical purposes. The amount of formaldehyde gas furnished by one "standard size" candle is equivalent to about 1½ oz. formalin (40 per cent) when liberated with potassium permanganate, using Hill's 7–10 formula. The time required for the liberation in our experiment was 55 minutes.

Dr. Lowe's Solidified Formaldehyde, manufactured by The Dr. Lowe Formaldehyde Co., Chicago., Ill. is sold in two separate packages, the one containing a formaldehyde powder, the other crystals of potassium permanganate. The two sizes of this preparation were procured in the open market, one said to contain two ounces of formaldehyde, the other, four ounces. The two ounce size was accompanied by one-half pound of potassium permanganate and the four ounce size by one pound of potassium permanganate. The directions called for 40 to 45 ounces of hot water for the two ounce size and from 100 to 110 ounces of hot water for the four ounce size. The four ounce size was said to be sufficient to thoroughly disinfect 9,000 cu. ft. of space. On examination, the two ounce size was found to contain 38 gm. formaldehyde 96.6 per cent pure. The four ounce size was found to contain 77 gm. formaldehyde 96.6 per cent pure. One two ounce size and one four ounce size were used in our experiment together with the accompanying 1½ lb. potassium permanganate. To this was added 150 oz. of boiling water. According to the manufacturer's claims, this should be sufficient "to thoroughly disinfect 14,500 cu. ft. of space" but was employed by us in a relatively tight room of 3,000 cu. ft. The results in Experiment XXVIII can hardly be misinterpreted. A very violent reaction takes place between the permanganate and the formaldehyde, in which about 95 per cent of the formaldehyde is destroyed and the water evaporated. After seeing the chemical results, we were somewhat surprised at the bacteriological findings, which showed many of the cultures to be destroyed and this destruction was manifest in the dry as well as in the moist cultures. This is a marked contrast to some of the preparations liberating dry formaldehyde gas, where often over five times as much gas was found in the air without killing a single dry culture. While this preparation is worthless for practical disinfec-

TABLE 3.

EXPERIMENTS WITH PROPRIETARY ARTICLES.

Experiment Number	Name of Proprietary Preparation	Amount of Preparation Used per 1 000 Cu. Ft.	Temperature ° C.	Humidity Percentage	Amount of Formaldehyde in Mgm. Found per Cu. Ft. of Air Space	Cultural Results: + = Growth, o = No Growth, — = Not Killed in 5 Hrs. 48-Hr. Cultures. Exposed 5 Hrs. Incubation 96 Hrs. Number of Each Exposed, 3 Moist and 3 Dry. Staphylococci		Streptococci		B. coli		B. diphtheria		B. typhosus		B. anthracis		Control	B. coli First Culture Killed		Penetration B. coli Dry Culture
						Moist	Dry	Moist	Dry	Moist	Dry	Moist	Dry	Moist	Dry	Moist	Dry	Dry	Moist	Dry	1. Exposure 5 hrs. 2. Exposure 48 "
XV	Formanganate Disinfector	8 oz. Formalin	21	47	12.74	o	o	o	o	o	o	o	o	o	o	o	o	+	*20 m.	5 hrs.	1. None killed
XVI	DePree's Formaldehyde Fumigator	½ oz.	17	50	2.55	o	3+	o	3+	o	3+	o	3+	o	3+	o	3+	+	† 1 hr.	—	1. " "
XVII		1 "	18	73	5.09	o	3+	o	3+	o	3+	o	3+	o	3+	o	3+	+	*35 m.	—	1. " "
XVIII		1⅛ "	27	63	7.64	o	o	o	o	o	o	o	o	o	o	o	o	+	*40 "	—	1. " "
XIX		1½ "	22	86	7.00	o	o	o	o	o	o	o	o	o	o	o	o	+	*40 "	—	1. " "
XX		1½ "	23	65	7.22	o	o	o	o	o	3+	o	2+	o	o	o	o	+	*40 "	—	1. " "
XXI		2¼ "	23	72	12.10	o	o	o	o	o	o	o	o	o	o	o	o	+	*40 "	—	1. " "
XXII		3¼ "	20	56	16.35	o	o	o	o	o	o	o	o	o	o	o	1+	+	*40 "	—	1. " "
XXIII		3⅓ "	20	81	22.52	o	o	o	o	o	2+	o	o	o	o	o	1+	+	*45 "	—	2. " "
XXIV	International Germ Destroyer	1⅓ "	23	58	6.76	o	1+	o	1+	o	1+	o	3+	o	o	o	o	+	*40 "	—	1. " "
XXV		2½ "	26	56	11.47	o	o	o	o	o	o	o	o	o	o	o	o	+	* 1 hr.	5 hrs.	1. 2 layers killed
XXVI	Geo. Leininger's Formulas, Generator	2½ "	23	57	§8.92	o	o	o	o	o	2+	o	o	o	o	o	1+	+	*2 hrs.	--	1. None killed
XXVII	Lister's Fumigator	1⅔ "	23	40	2.55	o	3+	o	3+	o	3+	o	3+	o	3+	o	3+	+	* 1 hr.	—	1. " "
XXVIII	Dr. Lowe's Perm. Sol. Form.	1¼ "	26	60	.64	1+	2+	o	1+	2+	3+	1+	2+	o	o	3+	3+	+	—	—	1. " "

* First culture drawn after starting experiment. † Second culture drawn after starting experiment. § Air drawn 2 hrs. after starting experiment.

tion in the proportions recommended, our experience with it serves well to emphasize that oft repeated requirement for successful disinfection which is too often ignored, "An abundance of moisture."

TABLE 4.

SUMMARY OF PROPRIETARY ARTICLES EXAMINED.

Name of Preparation	Manufacturer	Amount Recommended Per 1,000 Cu. Ft.	Absolute Formaldehyde Contained	Method of Liberation	Approximate Loss in Liberation	Efficiency
Formanganate Disinfector	Parke, Davis & Co., Detroit	16 oz.	180 gm.	Permanganate (1–2)	40%	Efficient
DePree's Formaldehyde Fumigator	The DePree Chemical Co., Holland, Mich.	1 oz.	25 gm. Variable	Paraffin Candle	None Variable	Questionable
International Germ Destroyer	International Chemical Co., Chicago	1½ oz.	25 gm. Variable	Paraffin Candle	None Variable	Questionable
Dr. Geo. Leininger's Formaldehyde Generator	International Chemical Co., Chicago	1½ oz.	30 gm. Variable	Alcohol Lamp	None Variable	Questionable
Lister's Fumigator	Johnson & Johnson, New Brunswick, N.J.	Not Stated	45 gm.	Burning Product	75–80%	Inefficient
Dr. Lowe's Solidified Formaldehyde	Dr. Lowe Formaldehyde Co., Chicago	⅜ oz.	8½ gm.	Permanganate	90–95%	Inefficient

The percentage of formaldehyde in the air of the room on successive days seems to have been fairly constant for each method of liberation after a given time but shows considerable variation when influenced by weather changes for longer periods. Thus the percentage of gas recovered from the air after one hour varied during the time of our investigation as follows:

August 12–14—	Permanganate-formalin (1–2)	13.9 –14.16	per cent
August 17–21—	Permanganate-formalin (1–2)	17.78	" "
	DePree's Candle	30.27	" "
	International Candle	29.09	" "
August 24–September 28—	Permanganate-formalin (1–2))	13.1 –14.5	" "
	Permanganate-formalin (7–10)	14.7	" "
	DePree's Candle	25.75–26.55	" "
	International Candle	21.31	" "
September 30–October 9—	Permanganate formalin (1–2)	19.5	" "
	Permanganate formalin (7–10)	19.86	" "
	Permanganate-diluted-formalin Hill's formula	12.7 –12.9	" "
	DePree's Candle	35	" "

TABLE 5.

SUMMARY OF CHEMICAL RESULTS.

Preparation	Date, 1909	Temperature ° C.	Humidity Percentage	Wind Miles	Formaldehyde Product Used per 1,000 Cu. Ft.	Percentage Strength of Formaldehyde	Method of Liberation	Time Required, Minutes	Time Required to Draw Air	Absolute Formaldehyde Used per 1,000 Cu. Ft. in Gm.	Absorption per Sq. Ft. of Surface in Mgm.		Absolute Formaldehyde Found per Cu. Ft. of Space in Mgm.	Percentage Yield
											Moist	Dry		
Permang.-Form	9/23	17	85	7	15 c.c.	40	$KMnO_4$	5	1 hr., 5 m.	6	11	2	.85	14.10
Permang.-Form	9/28	20	66	15	30 "	37.8	$KMnO_4$	5	1	11	13	2	1.48	13.10
Permang.-Form	9/17	18	76	8	60 "	37.8	$KMnO_4$	5	1	23	15	6	2.97	13.17
Permang.-Form	8/19	26	70	8	120 "	37.8	$KMnO_4$	6	1–20	45	19	11	8.06	17.78
Permang.-Form	8/28	22	80	10	120 "	37.8	$KMnO_4$	6	1–10	45	23	11	6.58	14.51
Permang.-Form	8/12	21	44	2	240 "	37.8	$KMnO_4$	7	1–40	91	..	..	12.52	13.90
Fermang.-Form	8/26	23	82	6	240 "	37.8	$KMnO_4$	6	1–15	91	45	22	12.74	14.04
Permang.-Form	9/14	23	89	21	300 "	37.8	$KMnO_4$	6	1	113	29	19	15.50	13.67
Permang.-Form	10/7	21	75	6	300 "	37.8	$KMnO_4$	6	1	113	39	25	22.08	19.50
Permang.-Form	8/24	17	66	14	480 "	37.8	$KMnO_4$	6	1–15	171	63	43	25.05	13.80
Permang.-Dilut.-Formalin	10/2	19	73	4	330 "	37.8	$KMnO_4$	6	1	125	35	30	15.92	12.77
Permang.-Dilut.-Formalin	9/30	18	85	1	330 "	37.8	$KMnO_4$	6	1	125	53	24	16.15	12.96
Permang.-Form	9/25	18	79	18	300 "	37.8	$KMnO_4$	6	1	113	55	39	16.35	14.70
Permang.-Form	10/5	21	75	1	300 "	37.8	$KMnO_4$	6	1	113	45	27	22.50	19.86
Formanganate	8/14	21	47	7	240 "	37.8	$KMnO_4$	18	1–30	90	35	19	12.74	14.16
DePree's Candle	9/20	17	50	7	16 gm.	62	Paraffin Candle	30	1–10	10	22	6	7.64	30.27
DePree's Candle	9/21	18	73	11	31 "	62	" "	35	1–5	19	38	10	5.10	26.51
DePree's Candle	8/19	27	63	8	34.5 "	73	" "	38	1–30	25	16	6	7.64	30.27
DePree's Candle	9/2	22	89	3	44 "	62	" "	40	1–10	27	26	11	7.00	25.88
DePree's Candle	9/1	23	65	7	45 "	62	" "	40	1–20	28	32	11	7.22	25.75
DePree's Candle	8/31	23	72	4	72 "	63.6	" "	40	1–55	46	48	19	12.10	26.55
DePree's Candle	9/18	20	56	6	100 "	62	" "	40	1–15	62	54	20	16.35	26.37
DePree's Candle	10/9	20	81	4	104 "	62	" "	44	1	64	40	19	22.52	35.00
Leininger's Candle	8/21	23	58	2	40 "	57.9	" "	47	1–20	23	21	9	6.79	29.09
Leininger's Candle	9/4	26	56	5	77 "	69.6	" "	45	1–20	54	28	17	11.47	21 31
Alcohol Lamp	9/7	23	57	3	77 "	67.5	Alcohol Lamp	1–45	1–30	52	40	18	8.92	17.08
Lister's	9/9	23	40	2	50 "	91	Burning Product	55	1–5	45	9	2	2.55	5.60
Dr. Lowe's	9/11	26	60	7	38 "	96.6	$KMnO_4$	4	1–15	37	1	1	.64	1.72

The amount of gas recovered after one hour does not seem to have been materially affected by wind movements and the rate of leakage was about the same on a quiet as on a windy day. The amount of formaldehyde in the air of the room is practically in inverse proportion to the amount absorbed by the exposed surfaces, and if these surfaces be moist or contain a good deal of moisture, a larger proportion of the gas is absorbed than when the walls are dry and consequently less formaldehyde will be recovered from the air. An open vessel containing distilled water one-half inch in depth was found after 5 hrs. in Exp. XIII to have absorbed formaldehyde at the rate of 1.2 gm. per sq. ft. of surface.

CULTURAL RESULTS.

Abnormal variations in cultural results are to be expected, and no attempt should be made to interpret these except in a broad general way. McClintic[1] concludes "that in surface disinfection with formaldehyde, the organisms are mostly killed within the first hour of exposure if they are destroyed at all." In practical work it is not probable that any amount of formaldehyde which requires more than two hours for destruction is to be depended upon, for the rate of leakage from the average room in that time will have reduced the quantity of gas to a point where very little action can be expected. The rapid reduction in the quantity of gas present noted by McClintic, however, was probably due more to absorption than to leakage. It is a curious fact that the various proprietary manufacturers have in their possession testimonials and recommendations from numerous bacteriologists showing the efficiency of their product, and many of these bacteriologists are peculiarly men of national or international reputation. But the methods of investigation and the nature of the culture as well as the conditions of exposure vary so enormously that such testimonials are practically worthless. Dr. C. A. Marshall says: "To my mind, a silk thread that has just been moistened in the culture and exposed means very little. We are contending largely with desiccated forms, and when we come to the matter of determining the value of disinfectants in connection with desiccated forms, it has been

[1] *Hygiene Laboratory Bull. No. 27*, 1906.

my experience that there is no uniformity of results. For instance, I find that a thread that has been dried 24 hours will give quite different results than one that has been dried 72 hours." We have shown that a moist surface can absorb in thirty minutes 450 mgm. of formaldehyde per sq. ft., while a dry surface absorbed only 35 mgm. under the same conditions. The effect upon cultures is practically proportionate to the amount of moisture present. McClintic says: "The rôle played by the relative humidity in formaldehyde disinfection is more important than that of any other influencing factors. In the absence of moisture, formaldehyde is practically inert as a germicide." But he concludes that artificial moisture does not answer the purpose so well as the natural humidity of the atmosphere. McClintic however worked with cultures dried one hour, which are in reality moist cultures. Had he worked with thoroughly dried cultures, his conclusions would undoubtedly have been different. In our experiments, where 10 oz. or above of formalin was liberated by permanganate, the room became hazy with water vapor and invariably the B. coli cultures drawn after 15 minutes were incapable of growth. This water vapor we believe acted upon the cultures partially as did the distilled water used in the moist cultures. Dry cultures drawn into the room after it became clear were not killed even with much longer exposure. The result of our penetration experiments is highly unsatisfactory and no definite conclusions can be drawn. It would seem that formaldehyde cannot be depended upon for any marked degree of penetration and neither is there any special advantage in prolonging the exposure to more than eight or ten hours. The bacteriological tests used in each experiment given were duplicated by practical disinfection of two private houses and one suite of offices, using McClintic's (1–2) formula and 10 oz. forformalin for each 1,000 cu. ft. of space. In the office all cultures were killed exposed in different parts and the cultures in the penetration pad were killed after 30 hours through the fifteenth layer. In one of the houses five cultures under the first seven layers were killed, the remainder growing freely. In the other house with the penetration pad exposed in a bedroom none of the cultures in the pad were killed after 24 hours' exposure, but all cultures openly exposed showed no growth.

After considering the results of these experiments, we cannot but be impressed by the important advice of Werner[1] when he says:

1. The strength of the formaldehyde used should be known.

2. In exceptional cases, where numerous objects or a good deal of organic matter are present or where loss of formaldehyde cannot be avoided, the quantity of formaldehyde should be doubled.

It has been argued that under certain conditions a small amount of formaldehyde is sufficient. Such arguments are easily borne out by experimental evidence. In practical work, however, we are not dealing with certain conditions but with average conditions. It is evident that very small quantities of formaldehyde are sufficient to kill moist cultures of bacteria suspended in the air, but what practical value has this knowledge? Chapin has intimated that aerial disinfection is a myth and this idea is not without substantial foundation. Numerous able experimenters have shown the absence of specific pathogenic organisms from the air except as they are carried by floating particles of dust or other solid material. Bacteria are not winged insects flying through space, but solid bodies which fall by virtue of their own gravity. The surgeon gives little heed to the air which floats around his incision. But no skilled operator will plunge an instrument into his wound unsterilized, after dropping it on the floor. It is safe to estimate that one square inch of floor surface contains more bacteria under average conditions than the air of the entire room. Let us then turn our attention to the surfaces and be less considerate of the space. Surface disinfection is not a myth as can be easily demonstrated in the case of many infectious diseases. Rickards[2] has shown that B. tuberculosis may remain alive in sputum for at least three months. We have found B. diphtheria alive in membranes at room temperature after 48 days, and other observers have reported even longer periods. The virus of other infectious diseases has been shown to retain its virulence for a considerable period. The organisms to be reached in practical surface disinfection are not moist cultures suspended in mid-air, but organisms that have been dried for days or weeks, inclosed in whatever organic discharges they may have been excreted. Therefore the quantity of formaldehyde

[1] *Archiv f. Hyg.*, 1904, 50, p. 305.

[2] *Amer. Jour. Pub. Hyg.*, 1909, 19, p. 586.

must be sufficient to destroy thoroughly dried organisms and must possess sufficient penetrating power to reach them.

Formaldehyde has a powerful avidity for water. In the absence of water, even enormous quantities of formaldehyde exert but little influence. It has been argued that natural humidity only is of value at the time of disinfection and that artificially supplied moisture does not materially aid the efficiency of formaldehyde. For short exposures this is probably true. The high humidity we believe has only an indirect value in that it increases the amount of moisture in and on the objects to be acted upon, and with the increased moisture correspondingly more formaldehyde is absorbed.

What should be required for efficient room disinfection? Rickards says: "The amount of work involved in a proper solution of the problem is enormous, more than one man or a few men can hope to do."

McClintic concludes: "Formaldehyde gas, regardless of the method by which it is evolved, is a powerful surface disinfectant under certain conditions. Successful disinfection with it is so dependent upon several factors that its usefulness is more or less limited. The temperature and humidity are of primary importance, while the influence of the winds, character of the room, etc., are of secondary importance. A small quantity of formaldehyde is efficient for surface disinfection, provided the temperature and humidity are high. A large quantity assists penetration as also do high temperature and humidity, but even then the penetrating power of formaldehyde is so limited that it should not be employed for disinfection purposes requiring any marked degree of penetration. Depending upon the conditions as stated, the quantity of formalin may vary from 200 c.c. to 500 c.c. per 1,000 cu. ft. of air space." "For simplicity and rapidity the formalin-permanganate method is far superior to any other methods tried. It liberates the formaldehyde gas almost instantaneously and in almost as large a quantity as the retort and autoclave methods. The formalin-permanganate method is more applicable than any of the other methods for disinfecting an inclosure which is not comparatively tight."

For the purpose of ascertaining the methods recommended by other states, a circular letter, asking for information on various points,

was addressed to the secretary of the various state boards of health. Answers to this circular letter have up to the present time been received from 30 state boards of health, which answers are summarized as follows:

Formaldehyde disinfection is recommended by 29.

One does not believe in room disinfection.

Of the 29 which employ formaldehyde, 27 recommend the permanganate-formalin method, two recommed the retort method, injecting 1 lb. of formalin per 1,000 cu. ft. of space.

One uses the autoclave occasionally and three permit the use of solidified formaldehyde, one of these recommending the DePree candle in double the quantity recommended by the manufacturer, together with artificial moisture, the other two not specifying the method of liberating the gas.

One board considers the solid proprietary products all right for surface disinfection but does not believe they furnish sufficient penetration. Some of the boards are very emphatic in denouncing proprietary preparations. Artificial moisture is recommended by 24, and not recommended by 5, one considering it of questionable value.

In determining quantity, 24 consider space only, 5 consider surface as well as space, one believing this to be all important. Three increase the quantity when the temperature is below 60° F.

Among those recommending the permanganate-formalin method, four use 32 oz., one uses 20 oz., eighteen use 16 oz., three use 11 oz., and one uses 10 oz. per 1,000 cu. ft. of space.

The formulae employed for each 1,000 cu. ft. are as follows:

Permanganate 13 oz., Formalin 32 oz.—Four
Permanganate 7½ oz., Formalin 20 oz.—One
Permanganate 8 oz., Formalin 16 oz.—Seven
Permanganate 7½ oz., Formalin 16 oz.—Three
Permanganate 6¾ oz., Formalin 16 oz.—One
Permanganate 6½ oz., Formalin 16 oz.—Three
Permanganate 4 oz., Formalin 16 oz.—Two
Permanganate 3½ oz Formalin 16 oz.—One
Permanganate not stated, Formalin 16 oz.—One
Permanganate 11 oz., Water 9 oz., Formalin 11 oz.—Two
Permanganate 9 oz., Water 11 oz., Formalin 11 oz.—One
Permanganate 4 oz., Formalin 10 oz.—One

From the above replies we must conclude that the methods employed by the states are somewhat more satisfactory than in the cities investigated by Rickards, there being only one state which recommends a proprietary article of solidified formaldehyde and that being in not less than two ounce quantities. Two other states, however, recognize solidified formaldehyde without specifying method of liberation. This is very unsatisfactory, for, as we have seen, the

liberation of solidified formaldehyde may be by methods having little or no loss to a loss of 95 per cent of the product. We do not consider it necessary to buy the liquid preparation on analysis, for Liquor Formaldehyde is official in the U.S. Pharmacopoea and must contain at least 37.5 per cent formaldehyde. We made no inquiry concerning test cultures as a routine procedure in disinfection for we do not consider such tests as carried out by the average disinfector reliable, and the results that have been thus obtained are practically a worthless addition to our literature. The proportions of permanganate to formalin recommended by the various states is interesting, and employed as given, the amount of formaldehyde lost will vary from about 40 per cent with the 8 to 16 formula to about 75 per cent with the $3\frac{1}{2}$ to 16 formula. If the states using the lower proportions of permanganate would occasionally examine the residues remaining in the pail after a disinfection, they could hardly fail to appreciate the importance of increasing the permanganate. Any formula containing less than one part by weight of permanganate to two parts by volume of formalin is surely uneconomical, and with the present market values of formalin and permanganate the 7 to 10 formula of Hill is highly commendable.

THE BACTERIAL INTEGRITY OF COLLODION SACS.*

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The use of collodion sacs and parchment membranes for the study of the longevity of B. typhosus in natural waters and sewage was introduced by Jordan, Russell, and Zeit[1] in 1904 in their work on the Chicago Drainage Canal case. The particular advantages of this technic over the methods employed up to that time are that the typhoid bacilli are confined within comparatively narrow limits and can thus be recovered more easily than otherwise, and that at the same time the organisms thus confined are exposed to conditions as nearly as possible like those found in nature, since there is quite a free interchange of diffusible substances through the permeable walls of the sacs. In 1905–6 Russell and Fuller[2] used similar containers in a more extended study of the vitality of the typhoid bacillus in surface waters and sewage.

Recently the entire reliability of the results obtained by the use of collodion and parchment containers has been questioned on the ground that it is not possible to prepare either collodion or parchment sacs through which the typhoid organism will not pass in a few hours, and that the figures reported in the above-mentioned experiment are unreliable because they do not take into account the escape of a certain number of the typhoid bacilli through the walls of the sacs during their exposure in water and sewage. Johnson,[3] in a paper before the New England Waterworks Association, in 1905 first called attention to this, and cited certain experiments performed by him which apparently showed that B. coli could pass readily through parchment sacs, which retained perfectly their integrity as far as their dializing properties were concerned throughout the experiment. He showed that these organisms could be recovered from sterilized water in which parchment sacs filled with

* Received for publication June 28, 1910.

[1] *Jour. Infect. Dis.*, 1904, 1, p. 41.

[2] *Ibid.*, 1906, Supplement No. 2, p. 40.

[3] *Jour. N.E. Waterworks Assoc.*, 1905, 19, p. 506.

a starch solution and inoculated with B. coli had been immersed; also that B. coli appeared within sacs filled with water and sterilized, when such sacs were placed in sewage or polluted water. Johnson did not test the filtering properties of the sacs with B. typhosus, arguing that "if the less motile coli will pass through this membrane readily there is no room for reasonable doubt regarding the ability of the typhosus bacillus to act in a similar manner." This observer did not experiment with collodion membranes, however, and suggests that possibly this material "offers less opportunity for the exit of the highly motile typhoid bacilli through the walls of the sac than did the sacs of parchment."

More recently Todd[1] reports the result of some experiments on bacterial integrity of collodion and parchment membrane which seem to indicate that certain bacteria, notably those of intestinal origin, including the typhoid bacillus, dysentery, etc., pass readily through both collodion and parchment membranes of presumably perfect integrity. This observer tested "the growth of bacteria through collodion membranes" as follows: collodion sacs were made by Kellerman's[2] method and mounted on glass tubing. These were filled with broth and suspended in bottles containing broth. Air contamination was prevented by cotton packing and the complete outfit sterilized in the autoclave. Accidental contamination was guarded against by 24 hours' incubation in a thermostat. The sacs were then inoculated with the organism under observation and the outfit again placed in the thermostat. The passage of bacteria through the walls of the sac could be readily determined by the appearance of growth in the broth surrounding the sac. B. typhosus, B. dysenteriae, B. coli, B. aerogenes, B. cloacae, and organisms of similar character, also B. prodigiosus and B. pyocyaneus were recovered from the broth in which the inoculated sacs were immersed after a comparatively short incubation period. B. typhosus escaped from these sacs in 24 hours (average of eight tests), the shortest time being only four hours after inoculation. B. coli was recovered from the medium surrounding the sacs in 24 hours, B. prodigiosus in 24 hours (average of ten tests), and B.

[1] *Jour. Infect. Dis.*, 1909, 6, p. 368.

[2] *Jour. Applied Microscopy*, 1900, p. 2038.

pyocyaneus in 72 hours after inoculation. On the other hand, the non-motile cocci, B. subtilis, Bact. anthracis, and Sp. cholerae were retained in these sacs for several days. After the completion of the above experiments the sacs employed were retested by the air test, and if this was negative, by albumen solution.

"The direct passage of bacteria through collodion membranes" was tested by substituting distilled water for the broth used in the former experiment, thus removing conditions favoring growth. B. typhosus could be recovered from the water surrounding sacs inoculated with this organism in 14 hours, B. prodigiosus in 16 hours, and B. pyocyaneus in 27 hours. Todd also notes the passage of sewage bacteria through the walls of collodion sacs filled with sterile water and immersed in sewage, and in sterile water in which sacs filled with sewage had been placed. The earliest recovery of sewage organisms was made after 24 hours, and in no case did the membranes prevent the passage of these bacteria from the infected to the sterile liquid longer than 108 hours.

On the other hand, Frost,[1] in his studies on "The Antagonism of Certain Saprophytic Bacteria against B. typhosus," experienced little or no difficulty with collodion sacs which permitted the escape of organisms studied by him. The technic employed by him was very similar to that used by Todd. In many cases the medium in which the sacs were immersed was inoculated from one to six days before B. typhosus was introduced into the sacs themselves, so that the penetration of various saprophytic organisms into the sac could be readily detected. Sacs which became contaminated proved to be imperfect. Frost reports one experiment in which B. pyocyaneus was retained for a period of six months with a collodion sac, which was imbedded in gelatin instead of the usual broth. During this time the gelatin remained unchanged, altho luxuriant growth developed within the sac. After six months' growth the sac was intentionally ruptured and the surrounding gelatin rapidly liquefied by the escaping organisms.

Zeit[2] reported that collodion sacs immersed in river water became coated on the outside with a slimy deposit in about five days, but that the integrity of the sacs was not affected by it for several weeks.

[1] *Jour. Infect. Dis.*, 1904, 1, p. 599. [2] *Ibid.*, 1904, 1, p. 641.

In our hands the collodion sac method has given uniformly good results. Sacs filled with sterile water and immersed in water or sewage "showed no passage of bacteria through the sac membrane for a week or more."[1] While of course it is not possible to make perfect sacs at every trial, from 75 to 80 per cent of those made in this laboratory have proved entirely satisfactory. Furthermore, it is a simple matter to test the bacterial integrity of these containers by immersing them in polluted water or sewage for from 24 to 28 hours. Parchment membrane has not proved as satisfactory, for it is difficult to find sections of parchment tubing free from imperfection and of sufficient length to serve the purpose desired. For this reason parchment sacs were replaced by collodion containers in the latter series of experiments on the longevity of typhoid in water, in 1906.

With a view of proving that collodion sacs, similar to those employed in the typhoid studies referred to above, can be made so that they will remain unbroken for a period many times longer than that required to carry out the experiments cited, the following tests were made. Sacs were exposed to conditions most favorable to the passage of bacteria through the membrane, either by "growth" or "direct passage." The organisms tested were B. typhosus, B. coli, B. prodigiosus, and B. pyocyaneus and the bacteria of crude sewage and septic-tank effluent.

Sacs were made by the method recommended by Frost[2] in 1903, in test tubes 175 mm. in length and 25 mm. in diameter. The best results have been obtained from the use of a 10 per cent solution of Schering's collodion, in equal parts of absolute alcohol and ether. The sacs were air-dried for at least four or five hours before shrinking from the tubes with water. Air-drying for at least four hours is essential for the production of thin, tough membranes through which dialysis takes place most readily. Sacs dried for less than this time are frequently thicker and opaque, and dialysis takes place through them more slowly. After shrinking under water for several hours, the sacs could be slipped out of the tubes very easily, without sticking or tearing. The collodion tubes thus formed were trimmed to about five inches in length and slipped on to test tubes from which the bottoms had been cut, so that about three inches of the collodion sac protruded beyond the glass tubing. The sacs were tied firmly to the glass tubing with thread and the joints sealed tight with liquid collodion in order to exclude outside contamination. After filling the sacs with ordinary broth up to the supporting tube, they were plugged with cotton and suspended in Phillips beakers of 250 c.c. capacity, which were also filled with broth. These tubes were held firmly in

[1] *Jour. Infect. Dis.*, 1906, Supplement No. 2, p. 40.

[2] *Amer. Pub. Health Assoc. Papers and Reports*, 1903, 28, p. 536.

place by a tight packing of cotton around them in the neck of the beaker. All the dialyzing membrane was immersed in the culture medium which was protected from air infection by the cotton in the neck of the flask (Fig. 1, Sac B). The whole outfit was sterilized in an autoclave for 20 minutes at 15 pounds pressure. The sterilized containers and sacs were allowed to stand at room temperature for four or five days in order to be certain that no accidental contamination had taken place. The sacs were then inoculated with the organism to be studied. The inoculated sacs were kept at room temperature and daily observations were made. As long as the medium in which the infected sacs were immersed remained clear and showed no other evidence of growth, it was taken for granted that the bacterial integrity of the sacs had been maintained. When growth developed in the broth surrounding the sacs, tests were made to determine the character of the organisms causing the change in the medium. If the sacs retained their integrity for 60 days after inoculation, most of the experiments were discontinued and plate cultures made to recover the original organisms from the inoculated sacs and to prove the absolute sterility of the medium in which they had been immersed. A few experiments were allowed to run on indefinitely, and, at the present writing, June 20, there remain 12 sacs which have maintained their integrity for nearly six months. The results of these experiments are arranged in the following table:

TABLE I.

SHOWING LENGTH OF TIME COLLODION SACS RETAIN THEIR BACTERIAL INTEGRITY AFTER INOCULATION WITH VARIOUS ORGANISMS.

No. of Sac	Organism Tested	Date of Inoculation	Organism First Isolated from Medium Surrounding Sac
1	B. pyocyaneus	December 8	December 14
2	"	" "	" 16
3	"	" "	Not in 60 days
4	"	" 21	" " " " *
5	"	" "	" " " "
6	"	" "	" " " "
7	B. prodigiosus	" 15	January 25
8	"	" "	Not in 60 days *
9	"	" "	" " " "
10	"	" "	" " " "
11	B. coli	" "	January 25 *
12	"	" "	Not in 60 days *
13	"	" "	" " " "
14	"	" "	" " " "
15	"	" "	" " " "
16	B. typhosus	" 18	January 31
17	"	" "	February 10
18	"	" "	Not in 60 days
19	"	" "	February 10
20	"	" "	Not in 60 days *
21	"	" "	" " " " *
22	"	" "	" " " " *
23	"	January 10	" " " " *
24	"	" "	" " " " *
25	"	" "	" " " " *
26	"	" 31	" " " " *
27	"	" "	" " " " *
28	"	" "	" " " "
29	"	" "	" " " "
30	"	" "	" " " "
31	Crude sewage and typhosus	" "	" " " "
32	" " " "	" "	" " " "
33	" " " "	" "	" " " " *
34	Septic-tank effluent and typhosus	" "	" " " "
35	" " " " "	" "	" " " "

* Sacs marked with a star remain intact at date of writing, June 20.

From the results above it will be seen that the majority of collodion sacs tested retained their bacterial integrity for 60 days or longer. Six sacs were inoculated with B. pyocyaneus. Growth in the medium surrounding one of these developed in six days; the organism escaped from another in eight days. B. pyocyaneus was recovered in pure culture from the test broth in each case. The six-day sac had not been tightly sealed to the supporting tube and had slipped down into the broth surrounding it, so that the organisms escaped from the top of the sac rather than through the walls. This was proved to be undoubtedly the case, for after washing with distilled water this sac was sealed on to its glass support with fresh collodion and sterilized as before. The sac was reinoculated with B. typhosus and retained the organism for 60 days, when the experiment was discontinued. The eight-day sac proved to be defective. The four remaining sacs inoculated with B. pyocyaneus held perfectly for 60 days; three of them were discontinued but the fourth was allowed to run on and held germ-tight for 127 days. Subcultures from this sac showed actively motile bacilli, developing the deep-green pigment characteristic of B. pyocyaneus. The pigment of this organism diffuses readily through the walls of collodion sac and can be recognized in broth surrounding it within 36 hours after inoculation of the sac. After a month or more the medium becomes a very deep green but perfectly clear if the sac holds. The broth, in which the sac kept for 171 days was immersed, assumed a black color and was nearly opaque (Fig. 1, Sac I).

B. prodigiosus is also retained by collodion sacs for a considerable time. Of the four tests made, one sac held for 41 days and three over 60. Up to the time of writing one sac has held 174 days. Pure cultures of B. prodigiosus were obtained from this sac 170 days after inoculation (Fig. 1, Sac H).

Four out of five sacs, inoculated with different strains of B. coli, held for 60 days, and two sacs which were kept under observation longer retained the organism for 174 days. Pure cultures of B. coli were recovered from both these sacs (Fig. 2, Sac G).

Fifteen sacs were inoculated with B. typhosus in pure culture. Of these one held 49 days, two 59 days, and twelve over 60 days.

Eight of these sacs were kept under observation and still retain the organism with which they were inoculated at time writing (137–270 days) (Fig. 2, Sac F, Fig. 3, Sacs D, E).

In addition to the pure cultures used in these experiments, five sacs were filled with sewage and heavily seeded with a suspension

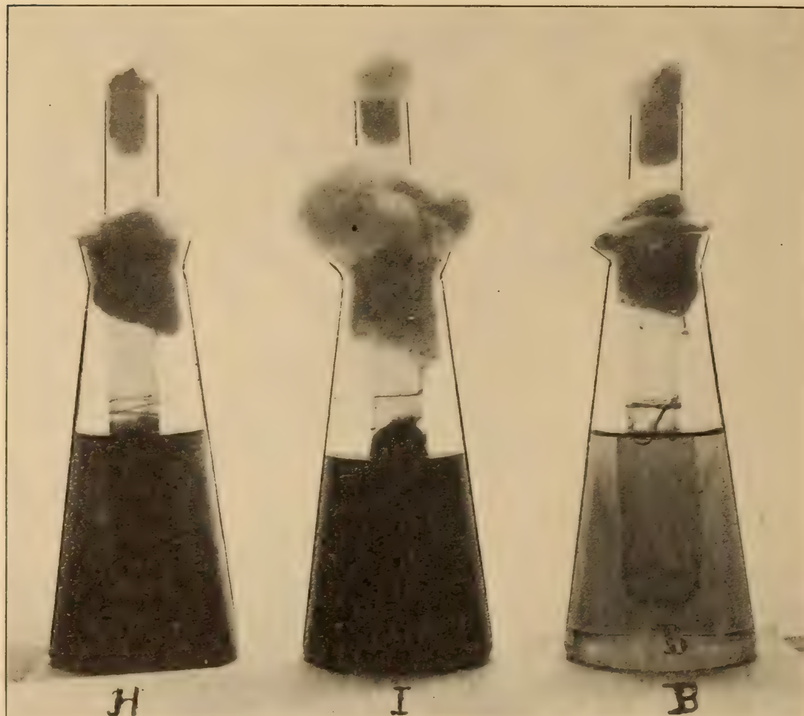

FIG. 1.—Photographs of sacs inoculated with B. prodigiosus and B. pyocyaneus. Photographs taken 71 and 65 days, respectively, after inoculation.

Sac H. Inoculated with B. prodigiosus December 15, 1909. The medium surrounding this sac assumed a red-brown color but is perfectly clear and the outline of the sac can be faintly seen within it.

Sac I. Inoculated with B. pyocyaneus December 21, 1909. The broth in which this sac was immersed became almost opaque and black in color from the diffusion of soluble pigments through the walls of the sac. The broth, however, remained perfectly sterile.

Sacs H and I. Retained their bacterial integrity at the time of writing, June 20.

Sac B. Control. Uninoculated sac in sterile broth.

of typhoid organisms. (After sterilization, the broth filling the sacs was carefully removed with a large sterile pipette and replaced with sewage.) Three sacs were filled with crude sewage, and two

with septic-tank effluent. All five sacs held 60 days and one (septic-tank effluent plus typhoid) retains its bacterial integrity at time of writing, 140 days after inoculation (Fig. 4, Sacs A, C).

In addition to the above experiments, two sacs were made in Erlenmeyer flasks of 400 and 250 c.c. capacity, respectively. The

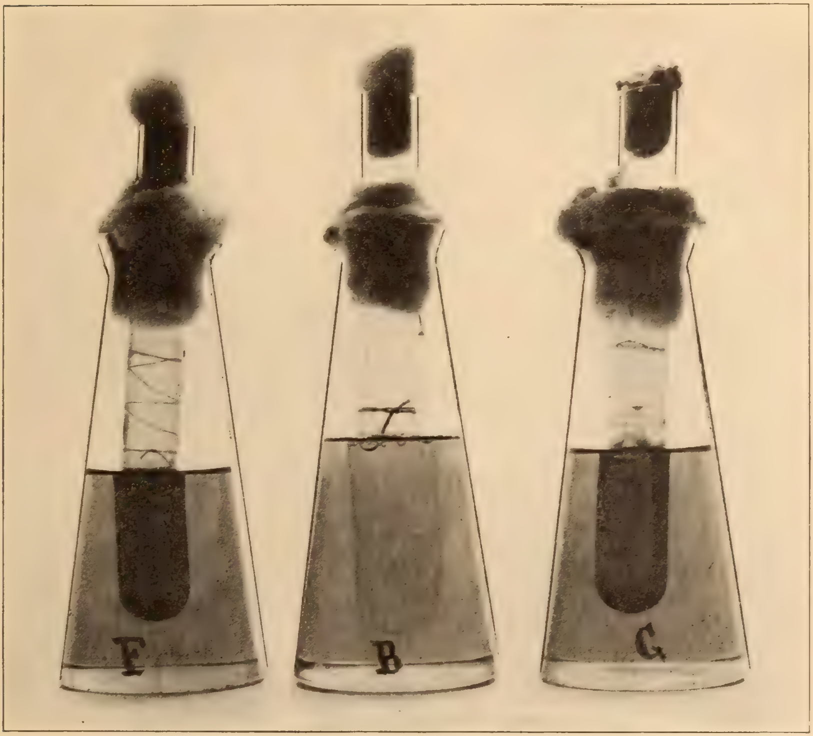

FIG. 2.—Photographs of sacs inoculated with B. typhosus and B. coli. Photographs taken 68 and 71 days, respectively, after inoculation.

Sac F. Inoculated with B. typhosus December 18, 1909.

Sac G. Inoculated with B. coli December 15, 1909.

The sacs showed very heavy growth and deposit at the bottom of the tubes, outlining the sacs sharply in the clear broth in which they were immersed. The beakers in which these sacs were suspended were sealed with paraffin, thus they do not show the evaporation noticed in Fig. 3, Sacs D and E. These sacs retained their bacterial integrity June 20.

Sac B. Control. Uninoculated sac in sterile broth.

400 c.c. collodion flask was sealed on to a glass tubing of slightly smaller diameter than the neck of the flask. This large sac was strong enough to support without rupture the weight of broth neces-

sary to fill it to the neck. This sac was immersed in broth in a 1,500 c.c. beaker, and held firmly in position by strong wire supports. A layer of cotton between two pieces of gauze covered the mouth of the beaker and protected the medium surrounding the sac from air contamination. The glass tube, supporting the sac, passed

Fig. 3.—Photographs of sacs inoculated with B. typhosus. Photographs taken 68 days after inoculation.

Sacs D and E inoculated with B. typhosus December 18, 1909. Note the accumulation of growth in the bottoms of these sacs; also the dry shrunken upper portion due to evaporation of the medium in the flask. These sacs are still germ-tight June 20.

Sac B. Control. Uninoculated sac in sterile broth.

through a hole in the center of the gauze and, after sterilization, a tight joint was made by sealing with paraffin. The whole outfit was autoclaved for 45 minutes at 15 pounds pressure. After sterilization the outfit was allowed to stand at room temperature for a week in order to insure against accidental contamination. The

sac was then inoculated with B. prodigiosus, maintaining its bacterial integrity for 61 days. During this time so much of the broth evaporated from the beaker that the sac finally ruptured, presumably from the weight of liquid and accumulated mass of organisms which filled the bottom of the sac to a depth of about

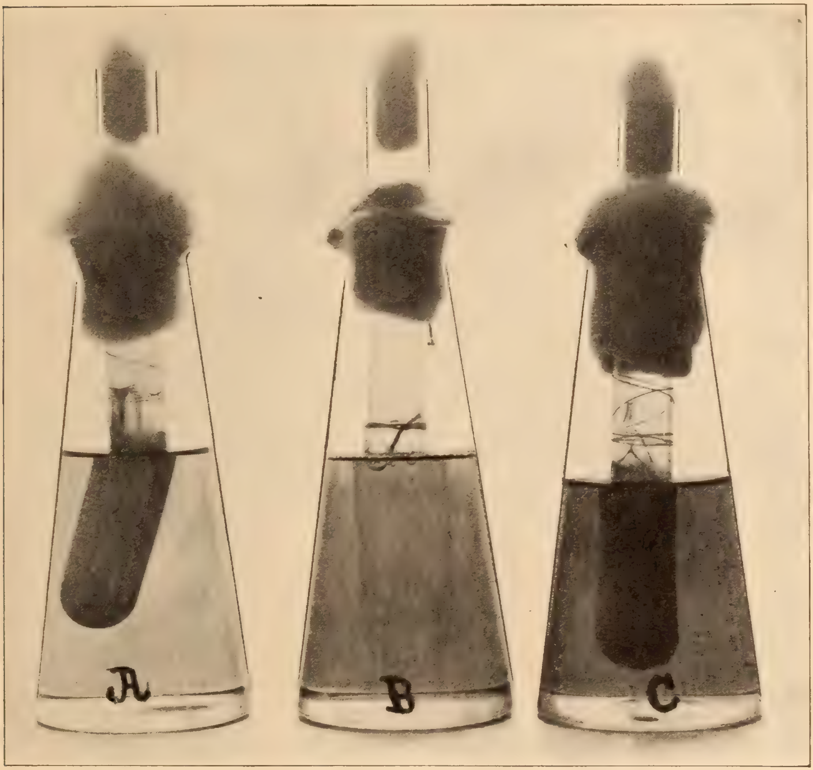

Fig. 4.—Photographs of sacs filled with crude sewage and septic-tank effluent and inoculated with B. typhosus. Photographs taken 24 days after inoculation.

Sac A. Filled with septic-tank effluent and inoculated with B. typhosus January 31, 1910. The sac became opaque on account of the very luxuriant growth which clung to the sides of it and is sharply defined in the sterile broth in which it is immersed. This sac is still intact June 20.

Sac C. Filled with crude sewage and inoculated with B. typhosus January 31, 1910. This sac also contains a very heavy growth.

Sac B. Control. Uninoculated sac in sterile broth.

half an inch. The sac formed in the 250 c.c. flask was mounted in a manner similar to the first. This sac was inoculated with B. typhosus and retained its integrity for 48 days.

The cultures of B. typhosus used in these experiments were sub-

cultures from a strain obtained from Parke, Davis & Co., and are used by the Wisconsin State Hygienic Laboratory for Widal determinations. The colon cultures were young and vigorous strains freshly isolated from feces.

CONCLUSIONS.

The results obtained from these experiments are entirely at variance with the results reported by Todd in 1909, and correspond perfectly with those obtained by us in former tests of the bacterial integrity of collodion sacs.

By Frost's method it is possible to make collodion sacs which will retain their bacterial integrity for several months. B. typhosus, B. coli, B. prodigiosus, B. pyocyaneus, and the bacteria of crude sewage or septic-tank effluent will not escape from sacs made by this method, either by "growth" or "direct passage through the walls."

The reliability of the results obtained by the use of collodion sacs for the determination of the longevity of B. typhosus in water and sewage cannot be seriously questioned on the ground that they do not take into account the escape of B. typhosus through the walls of the sacs during the coarse of these experiments.

THE VALUE OF COLLODION MEMBRANES AS FILTERS.*

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Most investigators have considered that the collodion membrane was only semipermeable, that it retained not only formed particles, but also to a greater or less degree soluble substances; and that according as these did or did not pass through the collodion sac some idea could be gained of the nature and size of their molecules. With this end in view, much work was done in filtering solutions of sugars, peptones, and proteins, and also of ferments, toxins, agglutinins, and lysins; the results obtained have however varied with the different investigators. The following experiments may explain these variations and assist in determining the value of the collodion membrane as a filter.

The sacs used in this work were made and mounted on glass tubes according to the Novy technic[1] with the modification of Gorsline;[2] they were about 2.5 inches in length and 0.5 inch in diameter. They were made by turning the tubes used twice in a dilute collodion; were without flaws or air bubbles, and of such thinness as to be practically invisible when placed in water; and they collapsed on emptying. After being mounted on glass tubes in a rubber stopper, they were placed in test tubes on a foot, provided with a side arm. The joint between the rubber stopper and the tube was air-tight. The sacs thus mounted were filled with distilled water, autoclaved at 105° for 15 minutes, allowed to cool, then emptied, immersed in sterile water, and subjected to air pressure (three inches of mercury). If there was no evidence of leakage, the filtration was commenced. This was carried on under a two-inch vacuum. After the filtration, the sacs were again emptied, immersed in water, and retested by pressure of three inches of mercury. If the sacs were still perfect, the filtrates were used for

* Received for publication June 25, 1910.

[1] *Laboratory Work in Bacteriology*, Ann Arbor, Wahr, 1899, p. 499.

[2] *Contributions to Medical Research Dedicated to Dr. V. C. Vaughan*, Ann Arbor, Wahr, 1903, p. 390.

inoculation experiments. In this manner diphtheria toxin and cobra venom were filtered.

DIPHTHERIA TOXIN.

Filtration of diluted diphtheria toxin.—The diphtheria toxin used was of such strength that 0.01 c.c. injected subcutaneously into a 250 gm. guinea-pig killed in 39 hours. The toxin was diluted 1 to 100 in 0.8 per cent sodium chloride. Three and one-half c.c. of this dilute toxin filtered through a collodion sac in 50 minutes. Whereas 1 c.c of this dilute toxin unfiltered killed a guinea-pig in 39 hours when inoculated subcutaneously, the same dose of the filtrate caused only a slight induration at the point of injection with complete recovery at the end of a few days. Repetition of this experiment gave the same results. In this instance the collodion filter had retained the greater part of the toxin, thus showing agreement with the results of Rodet and Guecheff,[1] who found that diphtheria toxin did not pass through a collodion membrane.

Filtration of undiluted diphtheria toxin.—The same toxin undiluted was filtered through a collodion sac. The filtration was much slower, as it took four hours to filter through 2.5 c.c. On diluting the filtrate 1 in 100 with 0.8 per cent sodium chloride, 1 c.c. was injected subcutaneously into a guinea-pig and caused death in 38 hours. Apparently all of the toxin had passed through

TABLE I.

FILTRATION OF DIPHTHERIA TOXIN THROUGH A COLLODION MEMBRANE.

Diphtheria Toxin	Subcut. Injec. in 250 gm. Guinea-Pig	Symptoms	Results
1. Dilute (1:100)	1 c.c.	Very slight induration	Recovery at end of 2–3 days
2. Dilute (1:100)	"	Induration	" " " " 5 "
1. Undiluted	1 " diluted 1:100	Death within 38 hours	
2. Undiluted	" " "	" " 4.5 days	
Unfiltered	" " "	" " 39 hours	

the collodion sac. On repeating this experiment, some of the toxin was evidently retained, as the same dose caused death, but after 4.5 days. Here, it will be seen, the results are at complete variance with those obtained with the diluted toxin. Evidently the concentration is an important factor in the question of the semipermeability of the collodion membrane.

[1] *Compt. rend. soc. de biol.*, 1900, 52, p. 955.

COBRA VENOM.

Filtration of dilute cobra venom.—A solution of cobra venom was made in 0.8 per cent sodium chloride, of such strength that each cubic centimeter contained 0.2 mg. of the venom. Four and one-half c.c. of this solution were filtered through a collodion sac in one hour and a half. One and one-half c.c. of the unfiltered venom (0.0003), injected intraperitoneally into a guinea-pig, gave rise to immediate symptoms of excitement, followed in 33 minutes by convulsions, and terminating in death in one hour and twelve minutes. But the same amount of the filtrate failed to cause any symptoms when injected into another guinea-pig; evidently the collodion membrane had not permitted the venom to pass through.

TABLE 2.
RETENTION OF DILUTE COBRA VENOM BY COLLODION MEMBRANE.

	Intraperit. Injec. in Guinea-Pig	Symptoms	Results
Filtrate.............	1.5 c.c.	No symptoms	
Unfiltered...........	" "	Symptoms; excitement at once; slight convulsions at end of 17 minutes	Death in 1 hour and 12 minutes

Dilution—0.0003 cobra venom in 1 c.c. 0.8 per cent NaCl solution.

Successive filtrations of dilute cobra venom.—Another solution of the cobra venon was made, in which each c.c. contained 0.8 mg. of the venom. Successive quantities of this solution were filtered through the same collodion sac. Four c.c. of the solution were put in a collodion sac. After 3 c.c. had filtered through, the filtrate was removed and labeled Filtrate 1; the fluid remaining in the sac was carefully pipetted off, and all moisture was absorbed from the outside of the sac by filter paper. Then a second 4 c.c. of the original solution were placed in the same sac, 3 c.c. again filtered through, marked Filtrate 2, and again the sac was emptied and partially dried as before. This was done because the fluid remaining in the sac was probably somewhat concentrated, as the water passes through most rapidly; and in this experiment it was desirable to keep the concentration of the fluid unchanged, previous work on diphtheria toxin having shown variations in concentration to be an important factor in the collodion filtration. In this manner four successive quantities of the cobra venom were filtered

through the same sac. The filtrations became successively slower. At the end of the filtrations, the sac as usual was emptied, immersed in distilled water, subjected to air pressure (three inches of mercury), and found to be free from leaks. The unfiltered venom and the first, third, and fourth filtrates were then injected intraperitoneally into guinea-pigs. The unfiltered venom caused symptoms of intense excitement at once; convulsions commenced within 5 minutes and the animal died within 31 minutes. The first filtrate gave rise to no symptoms within the first two hours, but the animal was found dead next morning. The second filtrate was not tested. The third filtrate gave symptoms of excitement at once, followed by convulsions and death in 58 minutes. The fourth filtrate gave immediate symptoms of excitement, convulsions within the first 10 minutes, followed by death in 43 minutes after the injection. This experiment shows the gradual passage of the venom through the collodion membrane, the filtrates varying from those in which the greater part of the toxicity was lost, to those which differed but slightly in strength from the unfiltered venom.

TABLE 3.

GRADUAL PASSAGE OF DILUTE COBRA VENOM BY SUCCESSIVE FILTRATION THROUGH THE SAME COLLODION MEMBRANE.

	Intraperitoneal Injection in Guinea-Pig	Symptoms	Results
1st filtrate (3 c.c.).....	1 c.c.	No symptoms of excitement or illness at once or in two hours	Found dead in 18 hrs.
2d filtrate (3 c.c.).....	Not tested		
3d filtrate (3 c.c.).....	1 c.c.	Symptoms of excitement at once	Death in 58 min.
4th filtrate (3 c.c.)....	"	Symptoms of excitement at once; convulsions at end of 10 minutes	Death in 43 min.
Unfiltered...........	"	Symptoms of excitement at once; convulsions at end of five minutes	

Dilution—0.0008 cobra venom in 0.8 NaCl solution.

SUMMARY AND CONCLUSIONS.

Dilute diphtheria toxin was retained by the collodion membrane, while the undiluted toxin passed through freely.

When dilute cobra venom was filtered, all toxicity was lost. On filtering successive quantities through the same collodion membrane, the filtrate gradually became toxic, until the fourth filtrate was practically of the same strength as the control. This result is

in accord with the work of Marbe[1] on the successive filtration of agglutinins through collodion sacs, and also with the gradual passage of complement through a Berkefeld filter as shown by me[2] and later found by Muir and Browning[3] working on the same subject.

Evidently filtration through collodion sacs, as through Berkefeld filters, is a phenomenon of adsorption, the substances in solution passing through when adsorption has reached a certain degree. Formed particles, however, if able to pass through at all, would pass through more rapidly in the beginning of filtration, and later, as the pores become clogged, they would be retained, while the opposite would occur with soluble substances, which appear in the filtrate only after adsorption has become more or less complete.

Thus, by changing the concentration, the quantity to be filtered, or the thickness of the sac, results may be obtained varying from total retention to complete passage of the active substances through the collodion membrane.

1 *Compt. rend. soc. de biol.*, 1909, 67, p. 809.

2 *Jour. Med. Res.*, 1904, 13, p. 409.

3 *Jour. of Path. and Bact.*, 1909, 13, p. 232.

NON-INHERITANCE OF IMPRESSED VARIATIONS IN STREPTOCOCCUS LACTICUS.*

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MICROORGANISMS should furnish unusually favorable material for a study of the immediate effect of environment upon cell characteristics, both physiological and morphological, and of the inheritance of the characters thus impressed. Bacteria in particular should prove to be valuable assets in the study of heredity on account of their rapidity of multiplication, the complete absence of the complications introduced by sexual reproduction, the relatively simple structure of the cell, and the intimate relationship necessarily existing between the protoplasm and the chemical and physical factors of the environment. These advantages are in part offset by the minuteness of the cells and the difficulty in isolation and recognition of individuals.

The present study was suggested by the considerable variation noted in the lactic acid production of the various starters used in the college dairy. The original purpose was to develop, if possible, a type of lactic acid organism that would produce an unusually large amount of lactic acid in milk, and to test the organism thus obtained in the commercial manufacture of butter. It was deemed advisable to use statistical methods as far as possible in recording the data obtained, both as an index of progress and for convenience in summarizing results. The need of such methods was particularly emphasized by the appearance of the papers of Goodman[1] on acid production in the diphtheria group and of Winslow and Walker[2] on the paratyphoid bacillus.

REVIEW OF PREVIOUS WORK.

The literature on the subject of variation in bacteria is voluminous, and numerous deductions as to the inheritance of characters may be found recorded. These latter in many cases will not stand careful analysis. In few instances have the data been

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† Mr. Truax made most of the culture transfers, titrated the greater portion of the samples, and assisted in the compilation of the data.

[1] *Jour. Infect. Dis.*, 1908, 5, p. 240. [2] *Ibid.*, 1909, 6, p. 90.

sufficient in quantity or of the right character to justify wholly the conclusions reached. The discussion here given of the work of others and of their conclusions is by no means exhaustive, as it has been thought best to include only the more noteworthy of the investigations and of the theories of heredity in microorganisms.

The work of investigators may for convenience be divided into studies on inheritance of morphological variations and on morphological mutants and into those that have to do with physiological variations. In few instances have careful statistical studies been made.

Beijerinck[1] secured an asporogenous race of Schizosaccharomyces octosporus by plating a pure culture and selecting those colonies in which no spores were to be observed. He found that the ability to produce spores did not return even after long cultivation under favorable conditions. Winogradsky[2] noted a similar loss of spore-producing power in Clostridium pastorianum when this organism was grown for some time on potato. Lepeschkin[3] succeeded in isolating races of Schizosaccharomyces pombe and S. melacei with a characteristic myceloid type of colony. These he considered to be true mutations in the De Vriesian sense, and not simply developmental stages in the growth of the organism. Later (1904) he reported a somewhat similar study on inheritance of branching in the cells of B. Berestnewi. Through selection he secured cultures in which the proportion of branched cells was greatly increased. He argues that a very direct relationship exists between the environment and the appearance of branching, and that cultivation in certain media tends to increase the amount of specific determinative material in the cell. This is then passed on its unusual quantities to the daughter cells. Hansen[4] by his work upon yeasts has demonstrated that the progeny of a single cell may develop into both top and bottom yeasts, and that selection may be used to fix this character. He also secured mutants, from the progeny of a single cell, that lost the ability to produce spores, and this power of spore production was not regained through cultivation under favorable conditions during many years. He believes the mutations observed to be brought about by definite changes in the environment. Barber[5] has shown that certain cells of the B. coli of unusual shapes, when isolated and cultivated, transmit the peculiarities in the few cases in which growth could be induced. These unusual shapes were not common, and many times failed to develop, but in several instances races were obtained from pure cultures that differed materially from the parent type. Garbowski[6] has contributed a most valuable statistical study of the immediate effect of environment upon the morphology of bacteria, particularly upon the cell dimensions. He gives no evidence, however, that these impressed variations may be transmitted. Jennings[7] has shown that in the Paramoecium there exists many pure races that remain true to type. He finds that mass selection may be used to modify mass characteristics, but this is accomplished by elimination of some of the races and not by the development of new characters. Changes in environment do not seem to modify permanently the morphology of a pure race, nor does continuous selection of individuals alter the

[1] *Centralbl. f. Bakt.*, Abt. 2, 1897, 3, pp. 449, 518.

[2] *Ibid.*, 1902, 9, p. 43.

[3] *Ibid.*, 1903, 10, p. 145; 1904, 12, p. 641; 13, p. 13.

[4] *Ibid.*, 1905, 15, p. 353; 1907, 18, p. 577.

[5] *Kan. Univ. Sci. Bull.*, 1907, 4, p. 3.

[6] *Centralbl. f. Bakt.*, Abt. 2, 1907, 19, pp. 641, 737.

[7] *Jour. Exp. Zool.*, 1908, 5, p. 577; *Amer. Nat.*, 1909, 18, p. 321; *ibid.*, 1910, 44, p. 136.

type. This work on a protozoan is a verification for these forms of the pure line theories developed by Johannsen in his work with plants. Clark[1] attempted to modify the morphology of various members of the diphtheria group; particularly did he try to convert the non-virulent type into the virulent. All his efforts failed to modify these characteristics in any degree. Each of the strains with which he worked maintained its own characteristic morphology with remarkable constancy.

There are numerous records of modifications of bacteria as to physiological and pathogenic characters. A review of the latter phase of the subject would scarcely be profitable. We have good reason to believe that virulence may be exalted or diminished in many organisms but by no means in all. Clark for example attempted to increase the virulence of the pseudo-diphtheria bacillus by repeated inoculations and isolations under what he believed to be the most favorable conditions, but he failed to modify the virulence to an appreciable degree. Color modifications have also been a favorite source material for studies of variation and inheritance in bacteria, with the most conflicting results. It seems entirely probable that in some cases true mutations and heritable modifications have been observed. Indol and gas production in members of the intestinal group and gas production in yeasts have been studied by various investigators (Peckham,[2] Horrocks,[3] Hartmann,[4] Massini,[5] Twort,[6] Burri[7]). It is difficult to estimate to just what extent the modifications observed by these writers are due to the selective action of environment on chance modifications or mutations, and to what extent to its influence on the mass of organisms. Careful statistical studies would seem to be required for this determination, for at present authors are by no means in agreement.

Goodman[8] selected a single colony of the diphtheria bacillus of a type producing an acidity of 2 per cent normal in dextrose broth, and made transfers to 15 tubes of sugar broth. The reaction of each tube was determined by titration at the end of three days, and a new series of 15 tubes inoculated from the tube showing the highest acidity and a similar series from those showing lowest acidity. Each of these, designated respectively the high and low series, was carried through 36 transfers. At the end of this time the maximum difference in reaction between the two series had risen to about 5 per cent of normal acid, and the difference in the means to $3\frac{9}{10}$ per cent. Goodman regards this as evidence that gradual modifications in the physiological characteristics may be cumulative and ultimately result in very considerable differences in the extremes. Winslow and Walker carried out a somewhat similar series of experiments in which they used two strains of the paratyphoid bacillus. The investigation "was planned so as to exclude the . . . . factor, the direct effect of environment and to deal with the inheritance of spontaneous variations of the fluctuating type." Each culture was plated and a hundred colonies isolated on agar, and from each agar tube one of 1 per cent dextrose broth was inoculated. Those tubes were chosen from which the culture showing the highest acid production in each series had been inoculated and from these new plates were poured, isolations made on agar and broth tubes inoculated from these as before, and their acidity determined. A third series was carried through in the same manner. The frequency polygons of each series were then plotted and compared. The selection seemed to be without observable effect, the polygons showed little or no evidence of change. These results in a certain measure may be opposed to Goodman's findings, altho the conditions of experimentation differed

[1] *Jour. Infect. Dis.*, 1910, 7, p. 335.
[2] *Jour. Exper. Med.*, 1897, 2, p. 549.
[3] *Jour. Roy. Army Med. Corps*, 1903, 1, p. 362.
[4] *Wchnschr. f. Brauerei*, 1903, 20, p. 113.
[5] *Centralbl. f. Bakt.*, Abt. 1, 1906, 38, p. 98.
[6] *Ibid.*, Ref. 1907, 42.
[7] *Ibid.*, Abt. 1, 1910, 54, p. 210.
[8] *Loc. cit.*

in two cases. In these cases there do not seem to have been intrinsic heritable variations, and it would seem that such variations must exist in the diphtheria group or must be impressed upon the organisms by environment, otherwise there would be nothing upon which selection could act.

MATERIALS AND METHODS.

The original purpose of this investigation, as has been stated, was twofold, first to develop, if possible, a high acid race of the Strept. lacticus for experimental work, and second, to study statistically the effect of selection upon acid production of the Strept. lacticus. Milk, buttermilk, cream, and commercial starters of various types were plated in deep litmus lactose agar in various dilutions to obtain the source cultures for the work. These were incubated at blood heat and the acid colonies isolated as they appeared. Colonies of Strept. lacticus only were isolated. Altogether 20 cultures from as many different sources were selected for the work. The lactose broth used was prepared at two different times, about 20 liters each time. Every effort was made to keep all the media as uniform in composition as possible. The customary procedure for the preparation of sugar-free broth from beef was followed. The broth was made neutral to phenolphthalein, autoclaved in liter flasks, and preserved for use as needed. Five per cent of lactose was added to these as required, and placed in test tubes and sterilized in the Arnold for 15 minutes on each of three successive days. The tubes after inoculation were kept in the thermostat at blood heat for three days and then titrated while hot with phenophthalein as an indicator against twentieth normal sodium hydrate. Blanks were kept with all cultures, and were titrated and their acidity (varying from 0.8 to 1 per cent normal acid) subtracted from the recorded acidities of the inoculated tubes. Plating was in all cases upon 1 per cent lactose agar.

TABULATION OF RESULTS.

Cultures of the Strept. lacticus from 20 sources were secured, as has been stated, by the isolation of colonies developing upon the litmus lactose agar. The diagnosis of the organism was made in every instance by its ability to coagulate milk, and by stained mounts. A transfer was then made of each culture to a tube of lactose broth, incubated for three days, and 5 c.c. titrated as above. Table 1 gives the results of this titration.

TABLE 1.

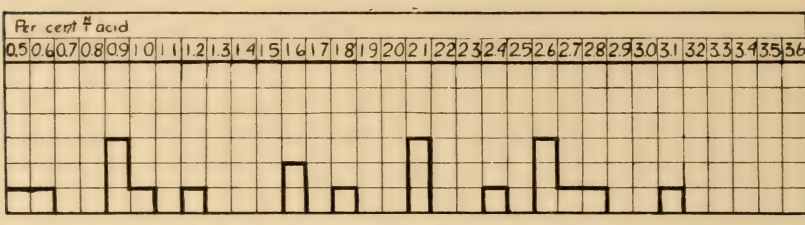
PERCENTAGE N/1 PRODUCED BY THE 20 ORIGINAL CULTURES OF STREPT. LACTICUS.

Culture	Percentage N/1 Acid	Culture	Percentage N/1 Acid
A..............	3.05	K...........	2.05
B..............	.94	L...........	1.64
C..............	2.71	M...........	2.61
D..............	1.79	N...........	No growth
E..............	2.64	O...........	.95
F..............	.48	P...........	1.64
G..............	2.81	Q...........	1.23
H..............	2.56	R...........	.90
I..............	2.05	S...........	2.35
J..............	.89	T...........	.62

The very considerable variation in the amount of acid produced is more clearly shown by the frequency polygon in Chart 1. In this series, as in all succeeding, the titrations were read to the second decimal place, but in preparing charts and tables the reading is usually to the nearest tenth.

The lowest of the series is F with 0.48 per cent N/1 acid and the highest A with 3.05 per cent. From each of these tubes plates

CHART 1.



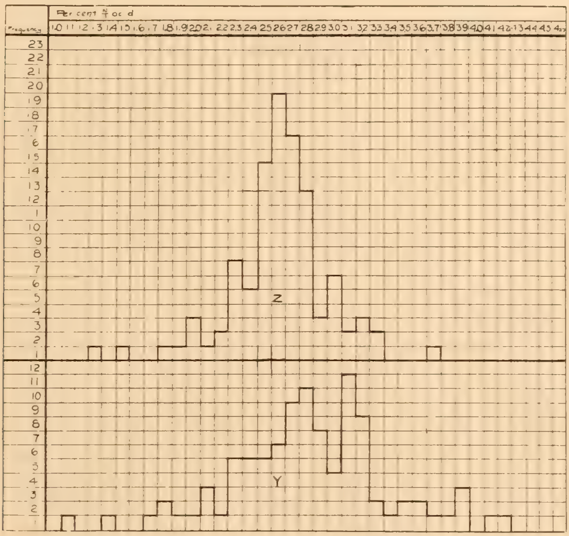
Frequency polygons for original isolations.

were poured in litmus lactose agar and incubated at 37° C. On the second day 100 tubes of lactose broth were inoculated from individual colonies, these were incubated, and titrated after 72 hours. This was done that a frequency polygon showing the distribution in acid production of individuals from each might be plotted for purposes of comparison and to serve as a standard that would record any subsequent deviation. For convenience in reference the high series is called Y and the low series Z. The first two columns of Table 3 give the distribution, mean and standard deviation, or index of variability for each series. Chart 2 gives the distribution of the population in each case in the form of a frequency polygon.

An examination of these figures and of the chart reveals an unexpected uniformity. The means differ by an appreciable degree 0.27 and the mean of the transfers from the high acid culture Y is higher than that from the transfers from the low acid culture Z. The difference is much less, however, than one might expect from the differences in the source cultures. It is noteworthy also that the lowest of either series is 0.6 per cent N/1 acid higher than the low original. Possibly too sudden changes in the osmotic

tension of the environing media may have inhibited growth to some extent in the low type, and equilibrium was not established sufficiently soon to allow normal acid production in three days in the original tubes. The highest and lowest acid cultures of each of these series were used for the inoculation in each instance of 10 lactose broth tubes. These were designated Low Y, High Y, Low Z, and High Z (abbreviated to LY, HY, LZ, and HZ). These were titrated after incubation and a new series of 10 tubes each

CHART 2.



Frequency polygons of initial low (Z) and high (Y) series

inoculated from the low acid tubes of the LY and the LZ, and from the high acid tubes of HY and HZ. These in turn furnished the basis for a new set. Transfers were made into 23 such sets of 40 in an endeavor to "breed" high and low acid races from Y and Z by continual selection of those cultures showing the desired characteristics. The method followed in its essentials was that of Goodman. Table 2 gives the results of these titrations.

To facilitate comparisons, the frequency polygons for acid production in these cultures have been plotted in Charts 3*a*, 3*b*, and 3*c*.

A study of these diagrams emphasizes two points in particular. First, changes in environment influence markedly the amount of acid produced. When cultures LY_5, HY_5, LZ_7, and HZ_7 were in the thermostat, the temperature fell to about 18° C. and an exami-

TABLE 2.

PERCENTAGE N/1 ACID IN LY, HY, LZ, AND HZ SERIES.

	LY_1	HY_1	LZ_1	HZ_1	LY_2	HY_2	LZ_2	HZ_2	LY_3	HY_3	LZ_3	HZ_3
	2.49	2.49	2.14	2.69	1.71	2.24	1.90	2.20	2.17	2.19	2.73	2.56
	2.17	2.21	2.69	3.01	2.80	1.80	2.72	1.21	2.05	1.79	2.77	2.24
	2.29	2.09	2.98	2.78	2.81	1.96	2.78	2.08	2.84	2.36	2.81	2.19
	2.40	2.65	2.85	2.70	2.07	1.95	3.06	1.37	2.65	2.45	2.78	2.12
	2.51	2.57	2.70	2.86	1.93	2.15	2.56	2.55	2.33	2.35	1.74	2.13
	2.25	2.29	2.65	2.56	2.01	2.33	3.00	2.54	2.57	2.06	2.62	2.16
	2.43	2.08	2.61	2.56	2.50	1.91	3.20	2.97	2.46	2.12	2.48	2.41
	2.79	2.68	3.20	3.55	1.38	2.30	3.20	2.98	2.44	2.20	2.65	2.70
	2.70	2.61	2.77	3.11	2.67	2.59	3.00	2.95	2.67	2.90	2.82	2.61
	2.69	2.42	2.73	1.98	2.53	1.79	2.80	2.92	....	2.21	2.78	2.55
Means......	2.472	2.409	2.732	2.680	2.241	2.102	2.822	2.377	2.464	2.263	2.718	2.367

	LY_4	HY_4	LZ_4	HZ_4	LY_5	HY_5	LZ_5	HZ_5	LY_6	HY_6	LZ_6	HZ_6
	2.17	1.92	1.91	2.30	2.29	2.16	2.41	2.80	2.27	2.15	2.69	2.93
	2.12	2.09	2.30	2.36	2.20	2.02	2.54	2.78	2.43	2.67	2.75	2.80
	2.38	2.36	1.98	2.40	1.70	2.00	2.54	2.62	2.49	2.70	2.83	2.84
	2.10	2.55	2.34	2.27	1.98	2.14	2.32	2.60	2.51	2.66	2.85	2.99
	2.30	2.68	2.16	2.36	1.95	2.16	2.70	2.49	2.59	2.82	2.92	3.02
	2.47	2.50	2.15	2.37	1.95	2.34	2.53	2.70	2.58	2.68	3.02	2.49
	2.28	2.39	2.01	2.00	2.15	2.43	2.55	2.78	2.32	2.52	2.73	2.68
	2.09	2.25	2.28	2.18	2.15	2.21	2.61	2.49	2.50	2.63	2.99	2.98
	2.32	2.33	2.20	2.00	2.02	2.10	2.40	2.90	2.51	2.57	2.67	3.13
	2.20	2.15	2.16	2.29	1.68	2.27	....	....	2.67	2.33	2.69	2.65
Means......	2.243	2.322	2.149	2.523	2.007	2.183	2.511	2.684	2.487	2.573	2.814	2.851

	LY_7	HY_7	LZ_7	HZ_7	LY_8	HY_8	LZ_8	HZ_8	LY_9	HY_9	LZ_9	HZ_9
	1.95	2.48	2.19	1.61	2.30	2.46	3.31	3.31	2.37	2.56	2.85	2.46
	2.48	....	1.79	1.63	2.38	2.68	3.24	3.24	2.65	2.89	2.89	2.30
	2.48	2.81	1.90	1.86	2.41	3.26	3.33	3.33	2.55	2.84	2.71	2.36
	2.39	2.44	1.81	2.09	3.01	2.88	3.14	3.14	3.07	2.68	2.52	2.88
	2.50	2.46	1.89	1.81	2.94	2.77	3.35	3.33	2.26	2.82	2.65	2.92
	2.12	2.45	1.86	1.99	3.24	2.25	3.31	3.31	2.87	2.99	3.02	2.99
	2.38	2.43	1.74	1.74	2.75	2.72	3.05	3.05	2.77	2.68	2.90	2.11
	2.26	2.58	1.97	2.09	2.63	2.55	3.09	3.09	2.88	2.92	2.90	2.55
	2.32	2.41	2.45	1.69	3.10	2.47	3.12	3.12	2.63	2.91	3.05	2.90
	2.60	2.65	2.58	2.13	2.78	2.65	3.16	3.16	3.00	3.08	2.95	2.71
Means......	2.348	2.523	2.018	1.864	2.754	2.664	3.214	3.208	2.705	2.837	2.844	2.618

	LY_{10}	HY_{10}	LZ_{10}	HZ_{10}	LY_{11}	HY_{11}	LZ_{11}	HZ_{11}	LY_{12}	HY_{12}	LZ_{12}	HY_{12}
	3.22	3.20	2.60	2.64	2.81	2.82	2.67	2.67	2.58	2.80	2.70	2.82
	3.05	3.17	2.65	2.52	3.24	2.43	2.70	2.67	2.47	2.67	2.83	2.83
	3.08	3.41	2.70	2.29	2.72	2.79	2.66	2.84	2.76	2.78	2.55	2.87
	3.18	3.34	2.65	2.37	2.98	2.69	2.74	2.75	2.94	2.95	2.71	2.80
	3.18	3.26	2.35	2.89	3.17	2.65	2.26	2.78	2.76	2.88	2.83	2.80
	3.33	3.03	2.47	2.62	3.15	3.11	2.53	2.64	2.75	2.92	2.76	2.90
	3.10	3.02	2.39	2.23	2.96	2.98	2.78	2.59	2.93	2.89	2.79	2.74
	3.00	3.37	2.40	2.86	3.09	2.72	2.62	2.44	3.38	2.84	2.55	2.55
	2.98	3.13	2.31	3.10	3.00	2.88	2.59	2.78	2.40	2.71	2.51	2.74
	2.77	3.30	2.85	2.70	2.98	2.55	2.54	2.79	3.01	2.95	2.88	2.66
Means......	3.089	3.223	2.537	2.622	3.010	2.762	2.609	2.695	2.798	2.839	2.711	2.771

TABLE 2.—*Continued.*

	LY_{13}	HY_{13}	LZ_{13}	HZ_{13}	LY_{14}	HY_{14}	LZ_{14}	HZ_{14}	LY_{15}	HZ_{15}	LZ_{15}	HZ_{15}
	2.22	2.48	3.05	3.05	2.64	2.55	2 50	3.15	2.59	2.57	2.50	3.00
	2.45	2.57	3.05	2.70	2.62	2.71	2.43	2.82	2.30	2.55	2.76	2.62
	2.89	2.85	2.96	2.91	2.71	2.80	2.94	2.80	2.46	2.50	2.69	3.02
	2.78	2.55	3.07	2.84	2.73	2.79	2.59	2.40	2.38	2.41	2.78	2.74
	2.99	2.56	2.94	2.80	2.77	2.60	2.88	2.25	2.56	2.17	3.07	2.87
	2.81	2.84	3.13	2.97	2.82	2.64	2.43	2.75	2.63	2.69	2.82	2.64
	2.85	2.50	2.66	2.88	2.90	2.56	2.69	2.50	2.63	2.29	2.77	2.73
	2.65	2.30	2.93	2.86	2.76	2.80	3.06	2.47	2.76	2.57	2.56	2.90
	2.84	2.72	3.16	2.94	2.68	2.58	2.88	2.49	2.52	2.73	2.33	2.75
	2.90	3.13	3.10	2.87	2.69	2.71	2.58	3.25	2.56	2.67	2.48	....
Means......	2.738	2.650	3.005	2.882	2.732	2.674	2.648	2.688	2.539	2.515	2.676	2.807

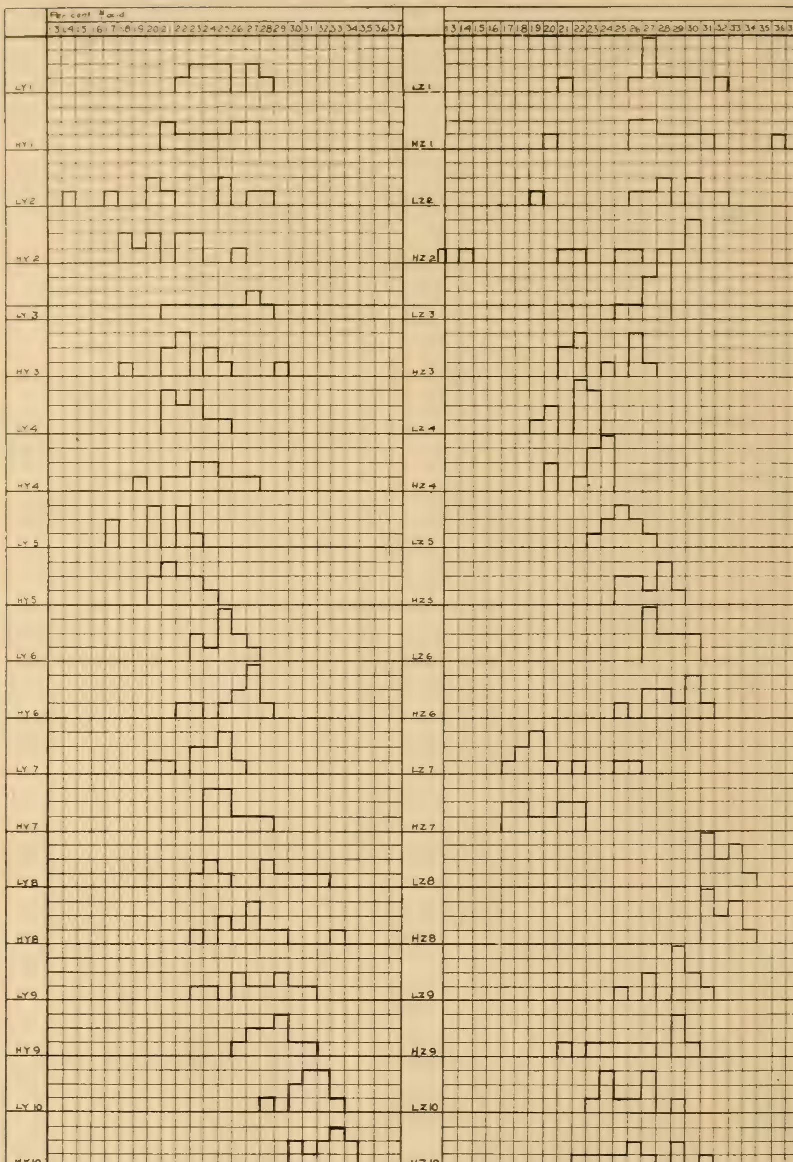
	LY_{16}	HY_{16}	LZ_{16}	HZ_{16}	LY_{17}	HY_{17}	LZ_{17}	HZ_{17}	LY_{18}	HY_{18}	LZ_{18}	HZ_{18}
	2.56	2.46	2.99	2.50	2.45	1.99	2.82	2.35	2.58	2.59	2.44	2.41
	2.46	3.26	2.92	2.51	2.88	2.71	2.49	2.34	2.54	2.53	2.70	2.60
	2.73	3.24	2.76	2.53	2.68	2.00	2.88	2.61	2.66	2.68	2.80	2.66
	2.67	3.05	2.45	2.40	2.84	2.95	2.10	2.94	2.70	2.81	2.82	3.06
	2.74	2.29	2.65	2.73	3.12	2.65	1.76	2.36	2.69	2.81	2.75	2.96
	3.04	2.44	2.86	2.77	2.77	2.78	2.67	2.19	2.32	2.63	2.64	2.96
	2.34	2.48	2.94	2.30	2.81	2.61	2.30	2.72	2.65	2.56	2.59	2.62
	2.61	2.56	3.00	2.40	2.46	2.74	2.43	2.89	2.31	2.63	2.70	2.81
	2.60	2.38	2.89	1.86	2.38	2.45	2.69	2.63	2.70	2.20	2.69	2.70
	2.70	2.41	2.80	2.83	2.47	3.00	2.61	....	2.53	2.60	2.81	2.63
Means......	2.645	2.657	2.826	2.633	2.686	2.588	2.474	2.626	2.568	2.604	2.694	2.741

	LY_{19}	HY_{19}	LZ_{19}	HZ_{19}	LY_{20}	HY_{20}	LZ_{20}	HZ_{20}	LY_{21}	HY_{21}	LZ_{21}	HZ_{21}
	2.20	2.90	2.74	2.70	2.46	2.49	2.84	2.45	2.34	3.03	2.43	2.58
	2.78	2.93	2.76	2.54	2.91	2.56	2.94	2.29	2.99	2.27	2.50	2.43
	2.73	2.66	2.77	2.64	3.02	2.82	2.35	2.67	2.83	2.63	2.61	2.90
	2.99	2.76	2.50	2.64	2.98	2.98	2.57	2.62	2.51	2.29	2.52	2.79
	2.69	2.82	2.64	2.77	2.67	2.84	2.83	2.89	2.38	2.69	2.45	2.70
	2.24	2.91	2.89	2.71	2.90	3.07	2.99	2.73	2.51	2.93	2.78	2.69
	2.53	2.81	2.77	2.83	2.95	2.60	2.89	2.45	2.85	2.76	2.64	2.90
	2.84	2.87	2.70	2.98	2.85	2.20	2.91	2.80	2.66	2.80	2.55	2.84
	2.68	2.41	2.97	2.65	2.96	2.80	2.55	2.99	2.57	2.83	2.71	2.86
	2.78	2.65	....	2.94	2.77	2.80	....	2.71	3.02	2.68	2.52	2.83
Means......	2.646	2.772	2.751	2.740	2.847	2.724	2.761	2.660	2.666	2.681	2.571	2.753

	LY_{22}	HY_{22}	LZ_{22}	HZ_{22}	LY_{23}	HY_{23}	LZ_{23}	HZ_{23}
	2.79	2.90	2.61	2.49	2.19	2.46	2.43	2.47
	3.09	2.87	2.80	2.77	2.72	2.82	2.83	2.68
	2.74	2.80	2.67	2.54	2.82	2.62	2.74	2.83
	3.06	2.62	2.63	2.75	2.68	2.63	3.07	2.93
	2.80	2.80	2.60	2.47	2.73	2.72	2.82	2.90
	2.78	2.39	2.60	2.74	2.75	2.70	2.92	2.95
	2.90	2.31	2.70	2.87	2.85	2.60	2.60	2.97
	2.99	2.80	2.81	2.86	2.90	2.66	2.79	2.54
	3.01	2.89	2.72	2.78	2.86	2.59	2.64	2.93
	3.07	2.41	2.88	2.69	2.99	....	....	2.73
Means......	2.919	2.679	2.702	2.696	2.749	2.642	2.484	2.794

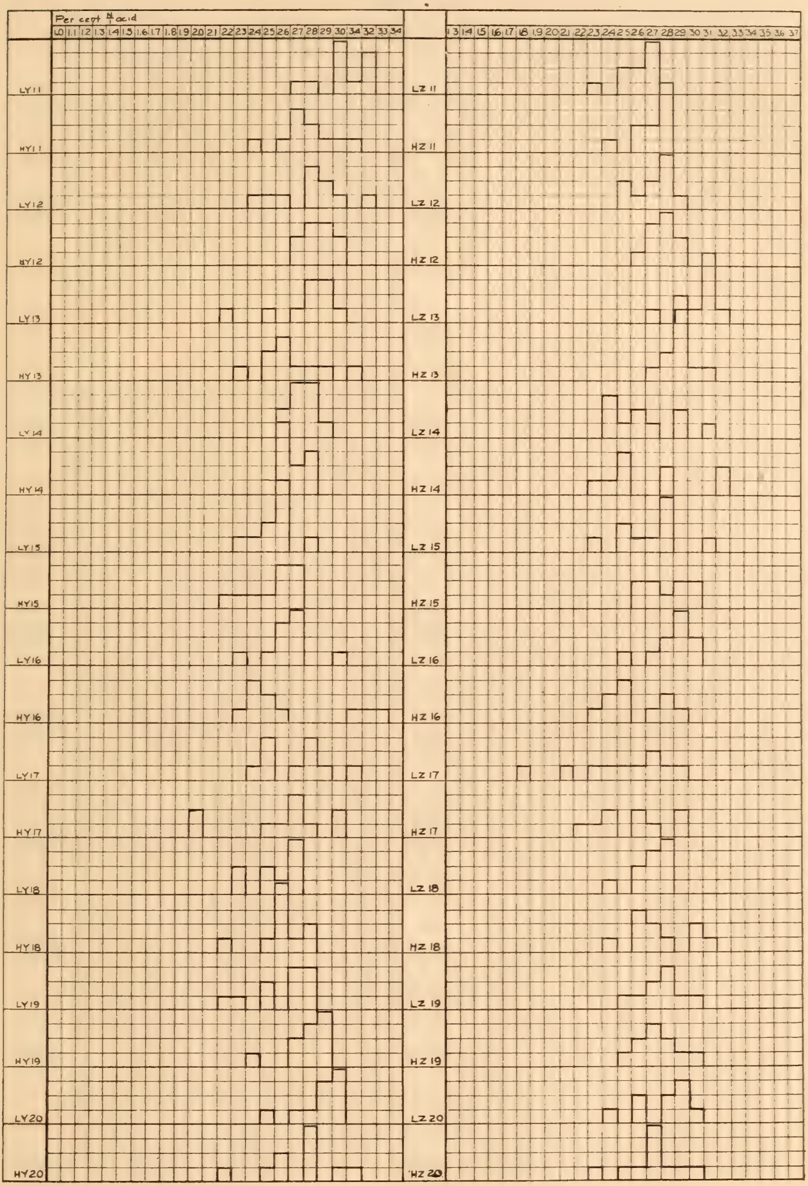
nation of the diagrams will show a considerable diminution in the amount of acid produced by these cultures. The next cultures show an increase again and a return to normal. At other times the temperature remained quite constant, yet there is to be noted a considerable variation in the amount of acid produced in the

CHART 3a.



Frequency polygons showing distribution in LY, HY, LZ + HZ series.

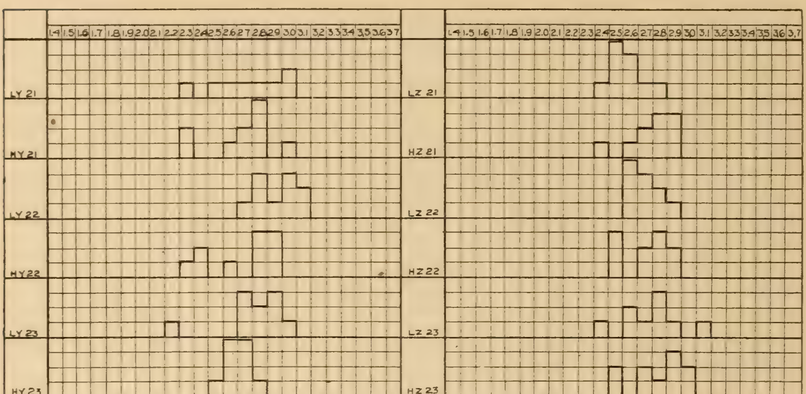
CHART 3b.



Frequency polygons of LY, HY, LZ and HZ series

various cultures. This is shown more clearly in Charts 4 and 5, where the maxima, minima, and means of each set of each series are plotted. None of the conditions of the experiment seem to account for the variation observed, altho it is undoubtedly due to some uncontrolled factors of the environment. Second, there is a very marked parallelism between the LY and HY series and the LZ and HZ series, and between the entire Y and Z series. Charts 4 and 5 show that there is a constantly close approximation of the means, maxima, and minima. There is certainly no such difference observable as would be expected if the series tended to diverge in

CHART 3c.

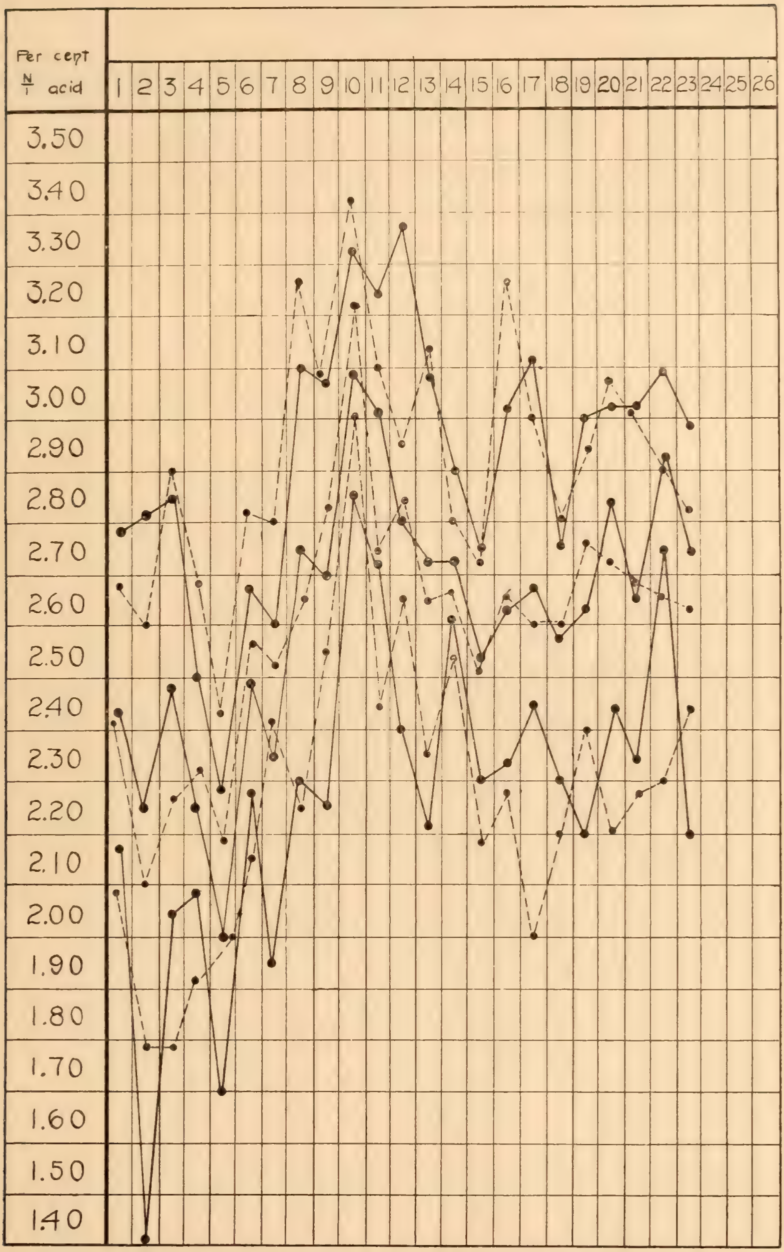


Frequency polygons showing distribution in LY, HY, LZ + HZ series.

the least. There is no evidence of any tendency toward the production of high acid and low acid races. Impressed variations of this type are evidently not heritable.

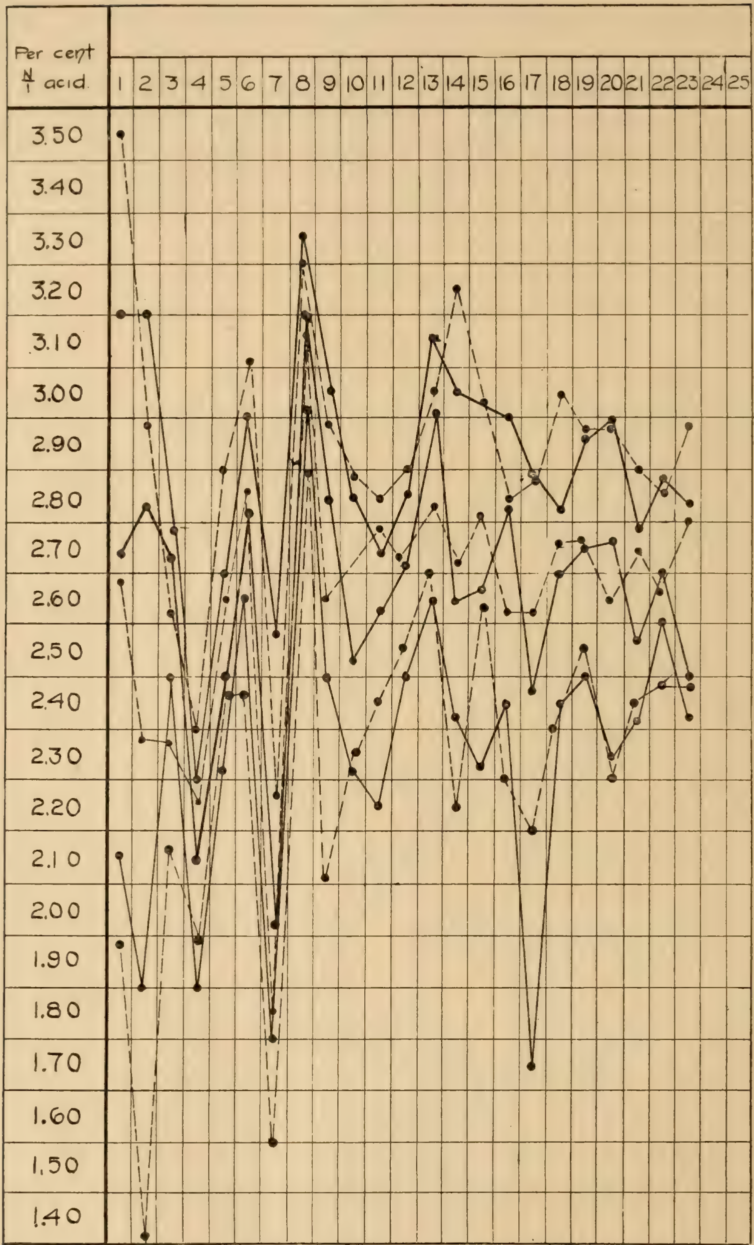
It was thought possible that 10 determinations for each series might not be a fair index to the true distribution of the acidities. To obviate this and also to secure cultures free from the immediate effect of the acid broth, litmus lactose agar plates were poured from certain members of each series. Such plates were in each instance poured from the tubes used to inoculate the next succeeding set of 10, that is, they were always from maximum or minimum acid tubes. From the plates from each tube 100 colonies were transferred to as many tubes of lactose broth, incubated, and acidity determined. Such isolations were made from the first, sixth,

CHART 4.



Maxima, Minima and Means of acid production in LY & HY series.
———— = LY. - - - - - - - - = HY.
Top – Maxima.
Middle – Means.
Bottom – Minima.

CHART 5.



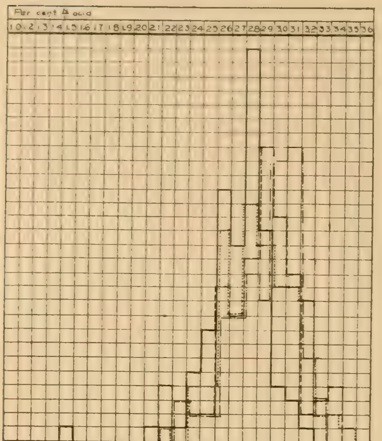
Maxima, Minima & Means of acid production in LZ & HZ series.
Solid lines LZ — Dotted Lines HZ.
Top – Maxima
Middle – Means
Bottom – Minima.

TABLE 3.

Percentage N/1 Acid	Frequencies																	
	Y	Z	YL_1	YH_1	ZL_1	ZH_1	YL_2	YH_2	ZL_2	ZH_2	YL_3	YH_3	ZL_3	ZH_3	YL_4	YH_4	ZL_4	ZH_4
1.0	..	..	..	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..
1.1	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
1.2	..	..	..	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..
1.3	..	1	1	2	..	..	..	..	1	..	..	..	..	..	..	..	..	..
1.4	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
1.5	..	1	..	..	..	..	..	..	1	..	..	..	..	..	..	..	..	..
1.6	..	..	..	3	..	..	..	..	..	..	..	..	..	..	..	..	..	..
1.7	1	..	..	..	..	..	..	..	1	..	..	..	..	..	..	..	..	..
1.8	2	1	..	2	..	2	..	..	1	..	..	..	..	..	..	..	..	..
1.9	1	1	..	2	2	..	..	..	1	1	..	..	..	..	..	..	..	..
2.0	1	3	..	..	2	..	1	..	2	..	..	..	..	3	..	..	..	..
2.1	3	1	1	3	3	2	4	..	1	2	1	..	1	2	..	..	..	..
2.2	1	2	1	1	5	2	..	2	7	3	..	2	1	4	3	2	..	1
2.3	5	7	5	9	9	4	2	3	2	10	4	6	4	7	2	1	2	1
2.4	5	6	8	5	15	8	2	4	5	12	4	1	11	9	2	6	6	4
2.5	5	14	18	12	20	13	11	8	14	14	15	6	14	12	8	11	10	3
2.6	6	19	14	21	24	23	9	10	8	23	9	14	18	11	14	18	17	13
2.7	9	16	28	13	10	20	13	20	7	15	17	18	22	19	12	18	13	19
2.8	10	12	15	12	6	13	20	20	8	11	14	14	12	12	10	14	22	20
2.9	7	3	4	6	2	5	15	18	10	..	11	18	9	7	20	22	15	20
3.0	4	6	3	3	2	6	11	13	2	..	11	9	4	9	21	8	11	11
3.1	11	2	1	..	..	..	2	3	..	..	10	3	2	4	6	..	3	5
3.2	8	3	..	1	..	..	3	..	..	..	3	4	1	1	2	..	1	2
3.3	2	2	..	..	..	..	4	..	..	..	2	1	2	1	..	..	..	1
3.4	1	..	..	..	..	..	..	..	..	..	1	..	..	..	..	..	..	..
3.5	2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
3.6	2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
3.7	1	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
3.8	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
3.9	3	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
4.0	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
4.1	2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
4.2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Total	97	101	99	97	98	98	97	101	73	91	102	96	100	101	100	100	100	100
Means	2.87 ±.036	2.60 ±.003	2.62 ±.015	2.48 ±.028	2.55 ±.016	2.61 ±.015	2.76 ±.018	2.75 ±.014	2.44 ±.028	2.53 ±.013	2.78 ±.017	2.73 ±.016	2.67 ±.017	2.64 ±.019	2.79 ±.012	2.71 ±.013	2.74 ±.013	2.79 ±.013
Standard Deviation	.5324 ±.0264	.3493 ±.0001	.2245 ±.0108	.4039 ±.0196	.2412 ±.0116	.2265 ±.0109	.2679 ±.0111	.2017 ±.0096	.3422 ±.0195	.1892 ±.0096	.2565 ±.0121	.2330 ±.0114	.2059 ±.0124	.2775 ±.0132	.1785 ±.0085	.1884 ±.0090	.1949 ±.0093	.1967 ±.0094

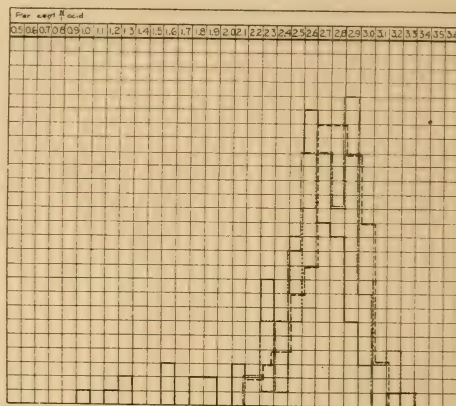
thirteenth, and twenty-third sets. Any tendency toward the inheritance of variations, impressed or mutative, should certainly be revealed by a comparison of results. The actual results deter-

CHART 6.



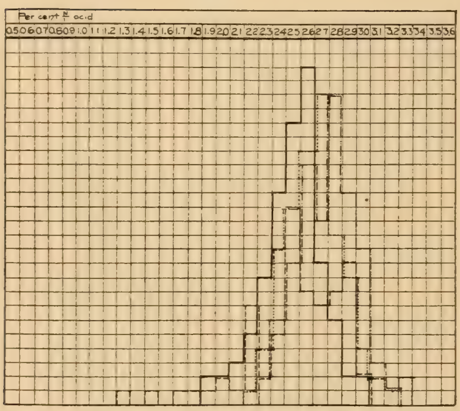
Frequency polygon for YL series
YL_1 = ——— YL_3 = ··········
YL_2 = – – – – YL_4 = — — · —

CHART 7.



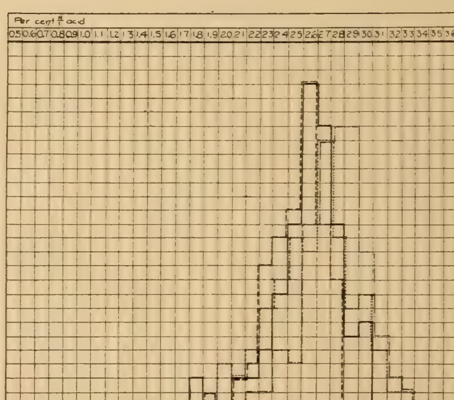
Frequency polygons for YH series
YH_1 = ——— YH_3 = ··········
YH_2 = – – – – – – – – YH_4 = — — · —

CHART 8.



Frequency polygons of ZL series.
ZL_1 = ——— ZL_3 = ··········
ZL_2 = – – – – – – ZL_4 = — — · —

CHART 9



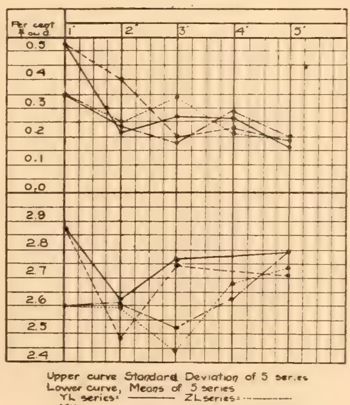
Frequency polygons of ZH series
ZH_1 = ——— ZH_3 = ··········
ZH_2 = – – – – – – – ZH_4 = — — · —

mined to the second decimal are not here recorded, but Table 3 gives the frequencies grouped in classes read to the nearest first decimal place. These series are designated YL_1, YH_1, ZL_1, and ZH_1 to YL_4, YH_4, ZL_4, and ZH_4, inclusive. The frequencies of

the original Y and Z series are also recorded. In one set, ZL_2, a part of the cultures were accidently destroyed, hence the total population of only 73 in this case. About 105 transfers were actually made in most instances, a few failed to grow, some others were lost through accidents so that in several other instances the total population recorded is less than 100. The means and standard deviations or indices of variation with probable errors are also here recorded.

The frequency polygons for the series given in Table 3 are plotted in Charts 6, 7, 8, and 9. A comparison of these curves shows that there is little or no tendency to a divergence of races. A comparison with the frequency polygons of the original Y and Z series plotted in Chart 2 indicates that there has been no material change from the original type.

CHART 10.



These four charts are summarized in Chart 10 by plotting the means and the standard deviations of each of the populations compared. In the diagram showing variation in means there is absolutely no evidence of a divergence of Y from Z or of LY from HY or LZ from HZ. The diagram plotting the curve of the standard deviations shows that the cultures are markedly less variable at the conclusion of the experiment than at the beginning. This may be due to a better habituation of the organisms to the medium in which they were grown, but no data are directly available on this question. The means and the standard deviations are also seen to approximate much more closely in the last transfers of the four series than in any of the preceding.

DISCUSSION.

The method here followed to test the inheritance of fluctuating and other variations is unsatisfactory in certain respects. There

is no way in which it can be determined whether heritable changes observed are due to mutation and the multiplication of the mutant and the crowding-out of the higher or lower acid forms by a gradual process of mass selection or whether changes observed are developed in all the individuals equally (mass variation). Burri believes the latter to be true with reference to indol production in the B. coli, altho his conclusions are not in agreement with other workers. Winslow and Walker obviated this difficulty by never subjecting the original cultures to the carbohydrate and acid environment, and by using the original cultures to determine mutations. The conclusions reached by them in the study of the two races of the paratyphoid bacillus agree very well with the result of the experiments recorded in this paper. Since no heritable variations could be induced in Strept. lacticus speculation as to methods and manner of variation is useless.

It should be noted that in all cases transfers were made from *acid* cultures to *neutral* broth, the organisms were therefore subjected alternately to favorable and unfavorable growth conditions. This work really amounted then to an attempt to secure two races of organisms respectively immune and susceptible to the deleterious influences of excreted metabolic products, and races that could withstand repeated transfers from a medium high in acid to another low in acid. We have no data that would determine with certainty whether or not the acid produced is the principal inhibiting factor in the growth of the organism, but it is very probable that it is such a factor. The problem of producing a high acid strain of Strept. lacticus might profitably be attacked by making repeated transfers to a medium having higher initial acid content. In this way it might be determined whether the acid-resisting powers of the lactic acid organism could be increased, and if so, whether or not acid resistance may be correlated with ability to produce acid. The differentiation of "mass change" from mutative changes would be a difficult problem, for the individual bacteria must needs then be isolated by plating out or by Barber's capillary pipette method.

Jennings compares the reproduction in protozoa to the dissolving of a crystal and to its recrystallization. "The young reappear in the form typical for the race without regard to the individual

peculiarities of the parent." While the former statement is scarcely true morphologically for bacteria as the cell wall is not resorbed during fission, nevertheless, the general statement may be said to hold, at least in the physiology of the bacteria.

SUMMARY.

An effort was made to secure high and low acid races of Strept. lacticus by constant selection and transfers of cultures from those tubes of lactose broth showing the highest and lowest acid content respectively. A comparison of these cultures carried through 23 transfers shows no evidence of a divergence of high and low acid races. These results were checked by isolation on plates from various cultures and plotting the acidity curves for populations of about one hundred. The results so obtained showed even more conclusively the fact that the variations observed are not inherited. There is no tendency to a divergence of the means. The cultures were distinctly less variable after a time, as indicated by a comparison of the standards of deviation.

It would seem that the simplest method of securing high and low acid races of Strept. lacticus would be to select from a great number of sources in an effort to secure such races already established. An effort to breed in the manner here outlined by selection would be futile. Jennings's statement, "We find that in a pure race of infusoria all the differences between individuals are environmental and without significance in inheritance," appears equally true with reference to the bacteria studied. The conclusion of Pearl and Surface, "It is found in actual experience impossible to bring about by selection improvement beyond the point already existing in the pure (isolated) strain," applies equally well to lactic acid bacteria.

CONCLUSIONS.

1. Selection as practiced under the conditions of this investigation failed entirely to fix high and low acid races of Strept. lacticus. Impressed variations do not appear to be heritable.

2. Continued growth of Strept. lacticus under favorable conditions seems to render the organism less variable.

OBSERVATIONS ON THE ANTI-INFECTIOUS POWER OF THE BLOOD OF INFANTS.*

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VARIOUS observers have compared the antibodies in the blood serum of the newborn with those in adult serum. Halban and Landsteiner[1] found that mother's blood possessed more hemolysins, agglutinins, bactericidins, antiferments, antitoxins, and precipitins than the blood of the fetus at birth. Wright and Douglas[2] found that the opsonic power of the serum of two infants at birth toward staphylococci equaled that of the mother. Wells[3] studied the opsonins of a large number of infants under one year old. Wells and Freeman made observations of the opsonic indices of the sera of parturient women and their newborn babies. The samples of the blood of the newly born infants were taken from the umbilical cord. The average opsonic indices of the blood of 17 babies as compared with those of 11 mothers were 0.8 to the tubercle bacillus, 1.18 to the staphylococcus, 1.4 to the streptococcus, and 0.39 to the colon bacillus. Pooling the sera of children from 1–4 weeks old and comparing their phagocytic index with that of the pooled sera of healthy men, Wells found that the opsonic indices to the tubercle bacillus, staphylococcus, streptococcus, and colon bacillus dropped considerably after birth. From the examination of individual samples of infant blood he found a rise in the opsonins later on and that the opsonic indices were subject to great fluctuations from time to time. He draws the following conclusions:

1. A low opsonic index is not diagnostic in children under one year old.

2. In infants a low opsonic index is not inconsistent with health, and the child may be thriving well with a declining index.

3. Where the opsonic index is low this will rise in response to the stimulus of an inoculation with a bacterial vaccine.

4. Results would appear to show that the healthy breast-fed infant possesses no advantages over the healthy artificially fed child.

5. The anti-bacterial defense in children cannot depend upon the opsonic content of the serum.

Amberg's[4] results using staphylococcus bear out those of Wells in regard to the drop in opsonic power after birth and the rise later on. Amberg found an increased opsonic power in the breast-fed infant, but considered it dependent to some extent on the state of nutrition of the infant and on its constitution.

Turton and Appleton[5] found in two 7- and 4-days-old babies that the opsonic power of their blood to the tubercle bacillus was much less than that of the mother's as compared with normal serum. They examined the staphylococcus opsonic index

* Received for publication May 10, 1910.

[1] *Münch. med. Wchnschr.*, 1902, 49, p. 473.

[2] *Proc. Roy. Soc.*, B., 1904, 74, p. 147.

[3] *Practitioner*, 1908, 80, p. 635.

[4] *Jour. Amer. Med. Assoc.*, 1907, 48, p. 304.

[5] *Brit. Med. Jour.*, 1907, 13, p. 865.

of the 7-days-old infant and found it also below normal. Much[1] observed that the staphylococcus and streptococcus opsonic index of the newborn was the same or only slightly less than that of adult serum.

Eisler and Sohma[2] studied the opsonic power of guinea-pigs toward staphylococci and of rabbits toward streptococci. They found that the blood serum of newborn animals possessed the same opsonic power as that of the normal mother. The opsonin appeared in the fetus during the last third of pregnancy. They did not find any transmission of immune opsonins from mother to child.

Flamini[3] studied the opsonic index in 26 normal infants, using rabbit leukocytes and the colon bacillus. The youngest babies examined by him were three days old. At this time he found that the opsonic power of the serum failed altogether or was small in amount as compared with normal adult serum and that it was not as great as adult serum even at the second year. He observed that the amount of opsonin varied with the weight of the child and was lower in artificially fed infants than in breast-fed.

Cathala and Lequeux[4] found that the staphylococcus opsonic index of the blood from the umbilical cord was perhaps a little lower than that of adult blood and diminished slightly during the first days of life.

No one appears yet to have studied the phagocytic power of infant leukocytes. As it has been shown that the phagocytic power of the leukocytes in health and disease varies quite as much as the opsonic power of the serum, I undertook to study the phagocytic power of the leukocytes as well as the opsonic power of the serum in normal infants.

These experiments concern healthy institutional children who had not been suffering from recent acute infections. Of those under one year all were breast-fed except two (one six weeks and the other four months old). On account of the small number of children examined, no light has been thrown on the influence of nutrition on the phagocytic power of the blood in infancy.

In order to compare the activity of adult and infant leukocytes, suspensions containing the same number of polymorphonuclear leukocytes were used. The washed leukocytes were standardized by counting the number of polymorphonuclear leukocytes in a given volume of each suspension by means of a white-blood counting apparatus. The leukocytic suspensions were then equalized by the necessary amount of normal salt solution.

Normal adult serum and leukocytes were used as the control. The control and infant blood were collected and kept under the

[1] *Münch. med. Wchnschr.*, 1908, 55, p. 572.
[2] *Wien. klin. Wchnschr.*, 1908, 21, p. 684.
[3] *Rivista di Chir. Pediat.*, 1908, 6, p. 572.
[4] *L'Obstétrique*, 1909, 14, p. 393.

same conditions as nearly as possible. The blood at birth was taken from the umbilical cord.

The bacteria employed in the experiments were streptococci, pneumococci, and staphylococci. The streptococcus was a typical Strept. pyogenes made virulent by passage through rabbits. The pneumococcus had been isolated from the blood of a pneumonia patient. The staphylococcus was a Staph. aureus isolated from a furuncle in a patient suffering from chronic furunculosis.

Mixtures containing equal volumes of serum, standardized leukocytes, and bacterial suspension were prepared in the usual way for determining the opsonic index. Mixtures were made of adult serum with both adult and infant leukocytes, and infant serum with both adult and infant leukocytes. From these four suspensions one can determine the influence on phagocytosis of (1) the infant's serum (opsonic index); (2) the infant's leukocytes (the cytophagic index of Glynn and Cox); and (3) the influence of the serum and leukocytes combined (the opsono-cytophagic index of Glynn and Cox[1]).

The opsonic index is obtained with both adult and infant leukocytes. The phagocytic activity of the leukocytes is estimated by comparing mixtures of *one* serum (adult or infant) and adult leukocytes with the same serum and infant leukocytes. The total phagocytic power of the blood is determined by comparing mixtures of adult serum and leukocytes with infant serum and leukocytes. This index undoubtedly gives a more accurate estimation of the phagocytic power of the blood than the opsonic index, because it includes both the opsonic power of the serum and the phagocytic power of the leukocytes. To obtain this index it is necessary to use washed standardized leukocytes. Using the whole citrated blood as suggested by Vietch[2] and others does not give the same results unless both specimens of blood contain about the same number of polymorphonuclear leukocytes.

[1] *Jour. of Path. and Bact.*, 1909, 14, p. 90. Glynn and Cox use the term cytophagic (an inversion of phagocytic) index to express the inherent phagocytic capacity of the leukocytes alone. They express the result of comparing the combined action of leukocytes plus serum of two persons by the term opsono-cytophagic index. This index gives the total phagocytic power of any blood. It corresponds to the "hemophagocytic" index of Shattock and Dugeon (*Proc. Roy. Soc.*, B., 1907, 80, p. 165), and the "opsono-phagocytic" index of Nikolsky. Nikolsky (*Centralbl. f. Bact.*, I. Orig., 1910, 53, p. 544) uses the term phagocytic coefficient instead of phagocytic index as usually employed to indicate the number of bacteria engulfed by the leukocytes of one person alone. He uses phagocytic index to denote the inherent phagocytic power of the leukocytes.

[2] *Jour. of. Path and Bact.*, 1908, 12, p. 353.

In two infants with diminished phagocytic power, counts were made to determine the variations in the number of cells belonging to the different classes of Arneth and their respective phagocytic power. No differences could be found between the adult and infant leukocytes so studied.

In the tables the indices are determined by estimating the number of bacteria ingested per leukocyte. Equivalent results are obtained by comparisons of the number of cells taking part in phagocytosis in the various mixtures.

Table 1 shows the opsonic indices of infant serum, using both adult and infant leukocytes. It indicates that the opsonic indices are about the same whether adult or infant leukocytes are employed.

Table 2 shows first the phagocytic activity of infant leukocytes (cytophagic index) using first adult serum and leukocytes and then infant serum and leukocytes as unity. The results are very similar whichever combination is used. The figures at the right end of Table 2 show the phagocytic power of the whole blood by comparing infant serum and infant leukocytes with adult serum and adult leukocytes (opsono-cytophagic index).

On finding that the serum of several infants at birth had the same amount of opsonin as adult serum and that the leukocytes also were as active as adult, while the infants examined after birth showed less opsonin and less phagocytic activity, the blood of two infants (marked (1) and (2) in the tables) was examined several times. The tables show that both the opsonin and the phagocytic power of the leukocytes in the two infants so examined decreased considerably a few days after birth.

The mothers of the children whose blood was examined at birth were apparently normal. Nothing in the previous history of the mothers of the first, second, and fifth babies in the tables could account for the differences between their blood and that of adult.

The blood of seven children who had recovered from various acute infections was examined. One seven-months-old baby who had just had an acute attack of enteritis had low opsonic indices to streptococci and staphylococci, being almost the same as those of a normal child of that age. The pneumococcus index in this child was normal. The leukocytes had less phagocytic power than the adult with

respect to all these organisms but somewhat greater than the leukocytes of a normal child of the same age. The other six infants had recovered from gonorrhea. These children, some in a bad state of nutrition, had opsonic indices which were quite as high as, sometimes higher than, those of infants of the same age who were apparently normal. The phagocytic power of their leukocytes also compared favorably with that of normal infants. Two children, two years old, who had recovered from gonorrhea had the same amount of opsonin as adult serum. One of these children had also recovered from diphtheria, and scarlet fever complicated with otitis media. Her leukocytes possessed the same phagocytic power as adult.

The seven- and eighteen-months-old normal infants had high opsonic indices to the pneumococcus both with adult and with their own leukocytes. Both children were from the same institution and were considered normal. The high pneumococcus indices indicate that they were unquestionably recovering from some pneumococcus infection and are therefore not included in the charts showing the opsono-cytophagic indices.

TABLE 1.

THE OPSONIC INDEX OF INFANTS' SERUM FOR STREPTOCOCCUS, PNEUMOCOCCUS, AND STAPHYLOCOCCUS.

Age	Sex	Opsonic Index of Normal Infants' Serum Using Normal Adult Leukocytes			Opsonic Index of Normal Infants' Serum Using Normal Infants' Leukocytes			
		Strepto-coccus	Pneumo-coccus	Staphylo-coccus	Strepto-coccus	Pneumo-coccus	Staphylo-coccus	Age of Infants' Leukocytes
At birth.....	B	0.4	1.1	0.88	1.4	1.0	0.9	At birth
At birth.....	B	0.6	0.67	0.29	0.9	0.6	0.8	" "
At birth (1)	B	0.79	1.2	0.96	0.9	1.2	1.3	" "
At birth (2)	B	0.77	1.3	0.8	0.99	1.2	1.1	" "
At birth.....	B	0.007	0.05	0.7	0.3	0.5	1.0	" "
At birth.....	B	0.4	0.02	0.4	0.3	0.01	0.3	" "
48 hours....	B	0.37	0.57	0.4	0.3	0.5	0.8	48 hours
5 days.....	G	0.94	0.05	0.3	0.9	0.6	1.0	5 days
5 days.....	G	0.91	0.08	0.4	1.3	0.8	....	5 "
6 days (1) .	B	1.1	0.39	0.6	1.6	0.5	0.4	6 "
6 days (2) .	B	0.3	0.5	0.19	0.32	0.7	0.7	6 "
11 days (2) .	B	0.26	0.5	0.66	0.7	0.7	....	11 "
11 days.....	B	0.64	0.16	0.8	....	....	....	....
13 days (1) .	B	0.46	0.5	0.24	0.17	1.0	0.1	13 "
6 weeks....	B	0.6	....	0.6	....	....	....	....
7 weeks....	B	0.3	0.0019	0.47	0.01	0.03	0.004	7 weeks
4 months	B	0.8	0.3	0.69	0.5	0.6	0.7	4 months
7 months	B	0.5	3.0	0.14	0.12	17.0	0.09	7 "
17 months...	B	0.6	0.8	0.25	0.6	0.36	0.2	17 "
18 months...	B	0.7	1.4	0.22	0.4	1.6	0.4	18 "
2 years.....	G	0.9	1.0	0.7	0.75	0.9	0.32	2 years
2 years.....	B	1.2	0.63	0.6	0.76	0.8	0.65	2 "
2 years.....	G	0.89	1.0	0.9	....	....	....	....
3 years.....	G	1.0	1.0	1.0	0.72	0.8	0.8	3 "

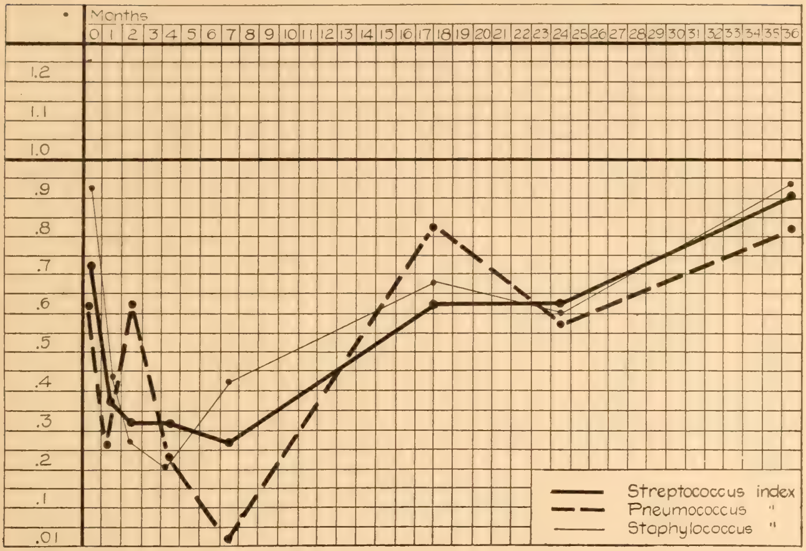

CHART 1.—The phagocytic power of infant leukocytes compared with that of adult leukocytes under the influence of adult serum (cytophagic index).

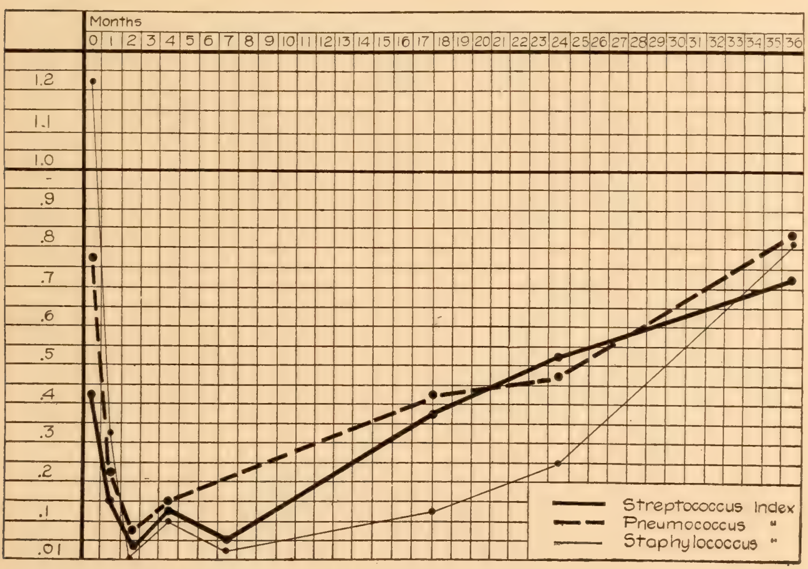

CHART 2.—The phagocytic power of infant serum and infant leukocytes compared with the phagocytic power of adult serum and adult leukocytes (opsono-cytophagic index).

TABLE 2.

The Phagocytic Power of Infants' Blood for Streptococcus, Staphylococcus, and Pneumococcus.

Mixtures	Phagocytic Index with Adult Serum			Cytophagic Index with Adult Serum			Mixtures	Phagocytic Index with Infants' Serum			Cytophagic Index with Infants' Serum			Opsono-cytophagic Index		
	Streptococcus	Pneumococcus	Staphylococcus	Streptococcus	Pneumococcus	Staphylococcus		Streptococcus	Pneumococcus	Staphylococcus	Streptococcus	Pneumococcus	Staphylococcus	Streptococcus	Pneumococcus	Staphylococcus
Adult serum and adult leukocytes..... Adult serum and leukocytes at birth...	4.0 3.7	1.34 2.3	1.8 3.2	0.9	1.7	1.7	Serum at birth and adult leukocytes..... Serum at birth and leukocytes at birth...	1.7 5.1	1.78 2.3	1.6 3.4	3.0	1.3	2.0	1.27	1.7	1.9
Adult serum and adult leukocytes..... Adult serum and leukocytes at birth...	2.4 1.4	1.9 0.8	3.0 0.9	0.5	0.4	0.3	Serum at birth and adult leukocytes .... Serum at birth and leukocytes at birth...	1.6 1.3	1.2 0.5	0.88 0.8	0.9	0.4	1.0	0.5	0.26	0.2
Adult serum and adult leukocytes..... Adult serum and leukocytes at birth...	1.9 1.69	4.9 4.4	2.7 3.6	0.83	0.87	1.3	Serum at birth and adult leukocytes..... Serum at birth and leukocytes at birth...	1.33 1.64	6.2 5.4	2.6 2.8	1.2	0.88	1.7	0.86	1.1	1.9
Adult serum and adult leukocytes..... Adult serum and leukocytes at birth...	2.2 1.9	2.1 2.1	2.6 4.4	0.8	1.0	1.6	Serum at birth and adult leukocytes..... Serum at birth and leukocytes at birth...	1.7 1.7	2.9 2.6	2.6 4.9	1.0	0.89	1.8	0.79	1.2	1.9
Adult serum and adult leukocytes..... Adult serum and leukocytes at birth...	1.3 0.3	2.4 0.18	3.4 1.7	0.2	0.075	0.5	Serum at birth and adult leukocytes..... Serum at birth and leukocytes at birth...	0.01 0.1	0.12 0.1	2.5 1.7	1.0	1.0	0.6	0.7	0.04	0.5
Adult serum and adult leukocytes..... Adult serum and leukocytes at birth...	1.98 0.44	3.5 0.25	1.68 0.82	0.22	0.07	0.5	Serum at birth and adult leukocytes..... Serum at birth and leukocytes at birth...	0.85 0.14	0.08 0.04	0.8 0.28	0.5	0.5	0.3	0.06	0.01	0.17
Adult serum and adult leukocytes..... Adult serum and 48-hour leukocytes (1)	2.1 1.5	4.1 3.2	2.2 3.0	0.7	0.7	1.3	Serum at birth and adult leukocytes..... Serum at birth and 48-hour leukocytes...	0.78 0.54	2.3 0.8	1.04 2.5	0.7	0.7	2.5	0.2	0.4	1.1
Adult serum and adult leukocytes..... Adult serum and 5-day leukocytes.....	4.1 0.96	2.4 0.5	1.7 0.64	0.2	0.2	0.3	Serum at birth and adult leukocytes..... Serum at birth and 5-day leukocytes	3.88 0.88	0.12 0.32	0.58 0.64	0.2	2.0	1.0	0.2	0.1	0.3
Adult serum and adult leukocytes..... Adult serum and 5-day leukocytes.....	6.9 0.72	3.7 0.2	4.4 0.48	0.1	0.05	0.1	Serum at birth and adult leukocytes..... Serum at birth and 5-day leukocytes	6.3 1.0	0.32 0.16	....	0.1	0.5	....	0.1	0.2	....
Adult serum and adult leukocytes..... Adult serum and 6-day leukocytes (1)..	7.9 1.8	10.56 0.32	4.36 0.68	0.2	0.03	0.1	Serum at birth and adult leukocytes..... Serum at birth and 6-day leukocytes	8.6 2.9	4.12 0.16	3.04 0.28	0.3	0.4	0.09	0.3	0.01	0.6

Adult serum and adult leukocytes.....	5.8	2.0	2.4	0.1	0.3	0.3	Serum at birth and adult leukocytes.....	2.1	1.0	0.45	0.1	0.5	1.2	0.06	0.26	0.2
Adult serum and 6-day leukocytes (2)	1.0	0.74	0.78				Serum at birth and 6-day leukocytes	0.32	0.53	0.59						
Adult serum and adult leukocytes.....	8.7	8.7	6.6	0.5	0.4	0.7	Serum at birth and adult leukocytes.....	2.2	5.0	....	1.4	0.5	....	0.3	0.3	....
Adult serum and 11-day leukocytes (2)	4.6	3.8	4.7				Serum at birth and 11-day leukocytes ...	3.2	2.8	....						
Adult serum and adult leukocytes.....	3.0	7.2	8.7	0.7	0.1	0.09	Serum at birth and adult leukocytes.....	1.38	3.9	2.1	0.3	0.3	0.07	0.13	0.13	0.18
Adult serum and 13-day leukocytes (1)	2.3	0.96	0.8				Serum at birth and 13-day leukocytes ...	0.4	1.0	0.15						
Adult serum and adult leukocytes.....	4.5	3.3	2.0	0.3	0.6	0.25	Serum at birth and adult leukocytes.....	1.6	0.06	0.94	0.001	1.0	0.002	0.004	0.01	0.006
Adult serum and 7-week leukocytes....	1.6	2.0	0.5				Serum at birth and 7-week leukocytes ...	0.02	0.06	0.02						
Adult serum and adult leukocytes.....	3.4	3.2	6.6	0.3	0.2	0.2	Serum at birth and adult leukocytes.....	3.0	0.96	4.5	0.2	0.2	0.2	0.16	0.1	0.14
Adult serum and 4-month leukocytes..	1.1	0.62	1.3				Serum at birth and 4-month leukocytes..	0.6	0.42	0.92						
Adult serum and adult leukocytes.....	3.8	1.3	6.0	0.26	0.03	0.4	Serum at birth and adult leukocytes.....	2.2	3.9	0.88	0.5	0.2	0.3	0.03	0.54	0.04
Adult serum and 7-month leukocytes..	0.98	0.04	2.6				Serum at birth and 7-month leukocytes..	0.12	0.7	0.24						
Adult serum and adult leukocytes.....	0.76	0.76	0.96	1.0	1.1	1.2	Serum at birth and adult leukocytes.....	0.44	0.68	0.24	1.2	0.4	1.0	0.7	0.4	0.24
Adult serum and 17-month leukocytes .	0.96	0.88	1.2				Serum at birth and 17-month leukocytes.	0.56	0.32	0.24						
Adult serum and adult leukocytes.....	3.8	1.3	6.0	0.2	0.5	0.15	Serum at birth and adult leukocytes.....	3.0	1.9	1.36	1.0	0.8	0.2	0.09	0.9	0.06
Adult serum and 18-month leukocytes.	0.72	0.76	0.9				Serum at birth and 18-month leukocytes.	0.32	1.26	0.36						
Adult serum and adult leukocytes.....	1.9	1.6	6.0	0.67	0.8	0.7	Serum at birth and adult leukocytes.....	....	....	....	....	....	....	....	....	....
Adult serum and 2-year leukocytes....	1.2	1.3	4.4				Serum at birth and 2-year leukocytes....	....	....	....						
Adult serum and adult leukocytes.....	2.1	1.7	4.5	0.7	0.4	0.7	Serum at birth and adult leukocytes.....	1.8	1.7	3.0	0.9	0.4	0.3	0.5	0.44	0.2
Adult serum and 2-year leukocytes....	1.4	0.8	3.0				Serum at birth and 2-year leukocytes	1.06	0.72	0.96						
Adult serum and adult leukocytes.....	2.1	1.7	4.5	0.6	0.6	0.44	Serum at birth and adult leukocytes.....	2.7	1.08	2.7	0.4	0.8	0.5	0.5	0.5	0.3
Adult serum and 2-year leukocytes....	1.38	1.16	2.0				Serum at birth and 2-year leukocytes	1.0	0.86	1.3						
Adult serum and adult leukocytes.....	2.4	2.1	5.5	0.87	0.8	0.98	Serum at birth and adult leukocytes.....	2.3	2.3	5.5	0.69	0.78	0.8	0.72	0.8	0.8
Adult serum and 3-year leukocytes....	2.1	1.7	5.3				Serum at birth and 3-year leukocytes	1.6	1.8	4.5						

The figures represent the average number of bacteria per leukocyte in mixtures of equal parts of human serum, bacterial suspension, and leukocytes.

The tables show considerable variation in the opsonic power of the serum and the leukocytic activity of infants of the same age. The charts are made from the averages of all the estimations for one age compared with adult leukocytes and serum.

Referring to the conclusion of Wells that low opsonic indices in children are not diagnostic, I would point out that to determine the opsonic index in infants their serum must be compared with that of healthy infants of the same age and not with that of adults. On account of the low opsonic indices in infants, Wells concludes that the antibacterial defense in children cannot depend upon the opsonic content of the serum. He does not seem justified in this conclusion in view of the fact that babies are so highly susceptible and show so little resistance to bacterial infections.

According to Holt,[1] 20–25 per cent of all deaths are of infants under one year; 55.1 per cent of the deaths at this time are caused by bacterial diseases. Holt shows that 25 per cent of the deaths of infants under one year occur during the first month of life, the death rate then gradually decreasing. My results, as seen from Chart 2, are in accord with these statistics, showing that during the first months of life when the death rate is highest the anti-infectious power of the blood is lowest.

It is unwarranted to draw definite conclusions from the small number of infants examined, but the results of my experiments would indicate:

1. That at birth the opsonic power of the blood serum toward streptococci, pneumococci, and staphylococci is a little less than that of adult serum. It falls still lower during the first months of life and does not equal the opsonic power of adult serum until about the second year.

2. That the phagocytic activity of the leukocytes of infants toward streptococci, pneumococci, and staphylococci follows a course similar to that of the opsonic indices. The leukocytes at birth are a little less active than adult leukocytes. Their activity diminishes considerably during the first months of life and does not reach that of adult leukocytes until about the third year.

3. The phagocytic power of the whole blood of infants drops

[1] *Jour. Amer. Med. Assoc.*, 1910, 54, p. 682.

decidedly during the first and second months of life and does not reach that of adult blood until about the third year.

4. During the first and second years of life the anti-infectious power of the blood as measured by the opsonic power of the serum and the phagocytic power of the leukocytes is far below that of adult blood.

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